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| (54) Title: POLYCHELANTS, THEIR COMPLEXES WITH METAL IONS, THEIR PREPARATION AND THEIR USES | | |
| (57) Abstract The present invention concerns a new class of polychelants, their chelates with metal ions and their physiologically acceptable salts, which can be used, either as they are or in association or formulation with other components, for diagnostic imaging as general or specific contrast agents for specific tissues, organs or body compartments. | | |

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POLYCHELANTS. THEIR COMPLEXES WITH METAL IONS. THEIR
PREPARATION AND THEIR USES

The present invention concerns a new class of polychelants, their chelates with metal ions and their physiologically acceptable salts, which can be used, either as they are or in association or formulation with
5 other components, for diagnostic imaging as general or specific contrast agents for specific tissues, organs or body compartments.

The new class of contrast agents is constituted by molecules or macromolecules obtained by covalently
10 linking chelants or chelates of metal ions to a "carrier" composed of an organic "backbone" which carries one or more primary amino groups to which said chelants/chelates are attached through alkylene bridges. This class is characterised by the fact that at least
15 one or, preferably, more primary amino groups of the "carrier" are bifunctionalised with alkylene residues carrying said chelants or metal chelates or their salts, while any remaining primary amino groups can be present either in a free form (salified or not) or
20 monofunctionalised with said chelant/chelate residues. This class of contrast agents usually contains a high number of chelant/chelate residues per molecule, which are attached to the primary amino groups present in the carrier. In fact, depending on the structure of this
25 carrier and the reactivity of said amino groups, up to two chelant/chelate residues can be attached to each primary amino group.

This invention concerns also a process for the

preparation of said molecules, as well as their uses..

Complexes formed of chelating agents and suitable metal ions are in use both in nuclear medicine and in magnetic resonance imaging (MRI). In nuclear medicine, radioactive metal chelates are used as both diagnostics (scintigraphy, PET or positron emission tomography) and therapeutics. In nuclear medicine, macromolecules with high biospecificity such as, for example, antibodies and more recently, polypeptides are widely used. In this latter case they are analogues (both agonists and antagonists) of biologically active polypeptides. An example of this approach is Octreoscan, a derivative of somatostatin carrying a complex of $^{111}\text{In}(3+)$, which was developed to visualise and localise tumours of neuroendocrin origin. A problem presented by these derivatives concerns the intrinsic biological activity of the carrier (also called address) macromolecules, the doses of which must be such as to provide improved visualisation of the organ under investigation, without inducing appreciable pharmacological actions. The possibility of increasing the number of diagnostically efficacious sites for carrier molecules would permit a reduction of the dose required to obtain the same diagnostic effect and therefore also a reduction of the possibility of undesired effects connected to the pharmacological activity of the molecule. This problem becomes more important when it is necessary not to modify a number of the amino groups, particularly those required for receptor recognition or biological activity. For example, it is known that the ϵ -amino group of lysine in position B29 of insulin can be

modified without compromising the biological activity, while none of the α -amino groups can be modified without altering the activity. The present invention, which enables the degree of substitution of the amino groups to be maximised, permits to get around this problem and thus is highly advantageous for diagnostic techniques characterised by low sensitivity, such as, for example, MRI.

In the preparation of biospecific contrast agents for MRI, the most common approach has been first to react macromolecules, such as proteins and polylysine, with chelating agents having functional groups capable of conjugating the ϵ -amino group of lysine, preferably through formation of amide or analogous bonds, and then to complex the resulting compounds with gadolinium (for ex. Ogan et al., Invest. Radiol., 1987, 22, 665-671). By this approach however it is not possible to link more than one chelant unit per amino group of the carrier. Indeed, the total number of chelant groups per protein is normally extremely low if compared with the total number of amino groups theoretically functionalisable on the molecule. For example, Lewis et al. (Bioconj. Chem., 1994, 5, 565-576) report the conjugation of the chelating agent DOTA (1,4,7,10-tetraazacyclododecan-N,N',N'',N'''-tetraacetic acid) to cytochrome c through activation with N-hydroxysulphosuccinimide. Increasing the molar ratio between the active ester of DOTA and cytochrome c from 10:1 to 100:1 results in increasing from 2.64 to 8.79 the average number of chelating groups attached to the protein, out of a total of 19 available primary amino groups.

In addition, the subsequent formation of the complex with gadolinium does not ensure that the same happens quantitatively. The overall result is that not all the amino groups are functionalised with chelating groups and that not all the chelating groups introduced become saturated with gadolinium. Neither does the state of the art teach to link the free amino groups of the macromolecules of interest directly with performed chelates, or their salts, in such a way as to obtain the maximum chelation possible. In this respect it is possible for example to cite the following documents: US 4,855,353, EP-A-481526, EP-A-243929, EP-A-255471, WO 9514491, GB 2169598 B, EP-A-038546, WO 9014881. As a consequence, diagnostically optimal doses of said contrast media contain high quantities of macromolecular carrier, with the result that undesired biological effects may arise. It would be highly desirable to be able to transport efficacious doses of metal chelate with substantially lower quantities of macromolecular carrier.

The present invention solves this problem by allowing the linking of up to two units of chelant, or, better, of its metal complex directly, to each primary amino group present on the carrier structure. This structure can for example be a macromolecule such as a protein, a polymer or a peptide, an amino acid or even a simple amine or polyamine. This aspect of the invention is particularly useful for those cases in which one or more primary amino groups of the address molecule must be maintained as such (for example after suitable, easily removable, protection) to preserve biological

activity or tissue organ specificity (see the case cited above for insulin). Also in these cases the dialkylation of other primary amino groups of the carrier with suitable chelant/chelate groups, according to the teaching of the present invention, yields a number of diagnostically or therapeutically active sites per molecule that is greater than that which is obtainable according to the current state of the art.

The same can be said for those polyamino carriers in which not all the primary amino groups undergo modification, because, for example, they are not equally sterically accessible. In this case also, the possibility of attaching two chelant/chelate residues per reactive primary amino group enables final products to be obtained that are more efficacious than those obtained by the current known methods. An example from the state of the art is given by the above cited patent application WO 9514491 which reports the preparation of diagnostic agents based on the 1:1 ion-pair Gd-DTPA-Lysine and dermatan sulphate (in the example of pag. 77), able to selectively visualise endothelial structures. Also in this case the ratio between chelant/chelate residues and the amino groups on the lysine are not greater than unity (there is in fact only one chelate group against two lysine primary amino groups). On the contrary, according to the procedure of the present invention, it is possible to obtain lysine derivatives at least with two chelant/chelate residues on one of the two amino groups, or even, two chelant/chelate residues for each one of the primary amino groups (for a total of four chelant/chelate groups

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against two amino groups, see examples 3 to 6 below), with the resulting advantages that:

- a) the quantity of dermatan sulphate necessary to obtain the same diagnostic effect is at least halved, as are, in consequence, also its anticoagulant effects,
- b) the resulting ion-pair is further stabilised by the high positive charge of the lysine derivative, since in this case the amino groups are not acylated (as in WO 9514491), but alkylated.

In addition, the present invention gives access to multiple Gd chelates that have a relaxivity r_1 which is increased over the value of the single chelates, also when calculated on the basis of the number of Gd ions. When a chelate is grafted to a macromolecule an increase in r_1 is expected; compare, for example, the myoglobin conjugate of Example 12, having $r_1 = 19 \text{ mM}^{-1} \text{ s}^{-1}$ on a per gadolinium basis, with the r_1 value for Gd-DOTA ($3.4 \text{ mM}^{-1} \text{ s}^{-1}$ according to the literature: e.g. Lauffer R.B. (1990) *Magn. Reson. Quart.* 2, 65-84). However, we show that it is also possible to prepare multiple Gd chelates of enhanced relaxivity and relatively low-molecular mass (see Example 6 for a lysine derivative, whose relaxivity has more than doubled from 3.4 of Gd-DOTA to $8.35 \text{ mM}^{-1} \text{ s}^{-1}$, on a per gadolinium basis). When this effect is combined with the increased number of chelated Gd ions a very high molecular relaxivity is obtained, with consequent substantial reduction of the efficacious dose.

Therefore the object of the present invention is a new class of polychelants/polychelates and their

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biologically compatible salts deriving from an organic backbone carrying m primary amino groups, where m is a number from 1 to 1000,

in which said amino groups are alkylated with n chelant/chelate residues, where n is a number from 2 to $2m$,

said chelant/chelate residues being covalently linked to said amino groups by means of an aliphatic chain, with this chain interrupted or not by heteroatoms selected from O, N, S, or by groups selected from carbonyl, thiocarbonyl, amide, ester, thiourea, and thioamide groups or aromatic groups, and in which a number p of said amino groups, where p is a number from 0 to $m-1$, is non-functionalised, said compounds being characterised by the fact that at least one of said primary amino groups is dialkylated with two of said chelant/chelate residues.

As a result, the number (n) of chelant/chelate residues on the molecule is always greater than the number of alkylated primary amino groups (which corresponds to $m-p$). In mathematical terms the situation can be expressed by the following disequation:

$$n > m-p ,$$

where m , n and p have the meanings described previously.

In other words, defining ρ as the ratio between the number of chelant/chelate residues inserted on the "carrier" and the total number of alkylated primary amino groups (mono and dialkylated), said ratio must always be greater than unity, that is:

$$\rho = \frac{n}{m-p} > 1$$

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Furthermore, since the maximum possible number of chelant/chelate groups per primary amino group is 2, ρ too cannot be greater than this value. We can say therefore that:

$$1 < \rho \leq 2.$$

This parameter ρ , is the main characteristic of the invention, together with the type of chemical bond that links the chelant/chelate residues to the primary amino groups of the carrier, and differentiates the same from the state of the art, where ρ is always equal to unity.

Are also a part of the present invention the complexes of the various chelants with the bivalent or trivalent ions of elements having atomic numbers between 20 and 31, or 39, 42, 43, 44, 49, or between 57 and 83 and their physiologically compatible salts. Particularly preferred are Fe(2+), Fe(3+), Cu(2+), Cr(3+), Gd(3+), Eu(3+), Dy(3+), La(3+), Yb(3+) or Mn(2+) or the ions of the following radioisotopes ^{51}Cr , ^{67}Ga , ^{68}Ga , ^{111}In , $^{99\text{m}}\text{Tc}$, ^{140}La , ^{175}Yb , ^{153}Sm , ^{166}Ho , ^{90}Y , ^{149}Pm , ^{177}Lu , ^{47}Sc , ^{142}Pr , ^{159}Gd , ^{212}Bi .

An object of the present invention are therefore polychelants or polychelates and their physiologically acceptable salts of formula:



in which

L is an organic backbone

F is a $-(\text{CH}_2)_q\text{-T-K}$ residue,

where

T is a simple bond or an aliphatic chain, interrupted or not by one or more heteroatoms selected from

O, N, S or by functional groups selected from carbonyl, thiocarbonyl, amide, ester, thiourea or thioamide groups or aromatic residues, said chain being linked covalently to a C, O, N or P atom of a residue K,

5

K is the residue of a linear or cyclic polyaminopolycarboxylic or polyaminopolyphosphonic or polyaminopolyphosphoric or polyaminopolyphosphinic chelant, or one of its metal chelates, or one of its salts,

10

q is an integer from 1 to 10,

p is a number from 0 to m-1,

z is a number from 0 to m-1,

x is a number from 1 to m,

15

where m is a number from 1 to 1000, being m the total number of the primary amino groups originally present on L,

with the condition that $p + x + z = m$,

and in which the chelated metal ions are bi- or trivalent paramagnetic ions or radioisotopes.

20

A first class of preferred compounds comprises those in which:

L is selected from the group constituted by: spermidine, spermine, norspermidine, 4,9-dioxadodecandiamine, 3,6-dioxaoctandiamine, ethanolamine and homologues, phosphatidylethanolamine and its derivatives, sphingosine, alkylamines, alkylenediamines, diethylenetriamine, triethylenetetramine, tris-(2-aminoethyl)amine, jeffamine, N-glucosamine, lysine and derivatives, ornithine, glycine, aminobutyric acid, aminocaproic

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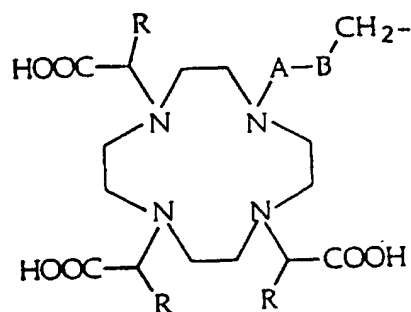
acid, taurine and its derivatives, [Lys³]-bombesin, insulin, chymotrypsinogen A, myoglobin, albumin, cytochrome c, branched and linear polylysine, branched and linear polyornithine, amino sugars, polypeptides, hormones, growth factors, antibodies;

T is a simple bond or an aliphatic chain, containing an ester, amide or carbonylamino group, said chain being linked covalently to a nitrogen or carbon atom of a K residue,

K is the residue of a polyaminopolycarboxylic acid selected from the group comprising: EDTA, DTPA, BOPTA, EOB-DTPA, DOTA, their derivatives, their metal chelates or salts,

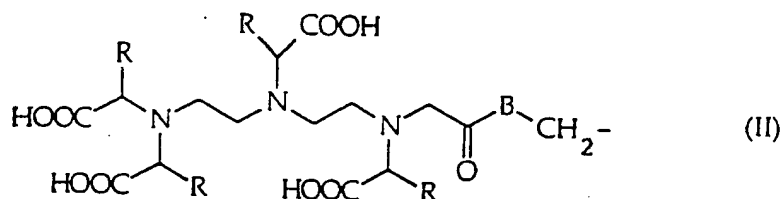
- the metal ion chelates are selected from the bi- or trivalent ions of elements having atomic numbers between 20 and 31, or 39, 42, 43, 44, 49, or between 57 and 83 or the ions of the following radioisotopes ⁵¹Cr, ⁶⁷Ga, ⁶⁸Ga, ¹¹¹In, ^{99m}Tc, ¹⁴⁰La, ¹⁷⁵Yb, ¹⁵³Sm, ¹⁶⁶Ho, ⁹⁰Y, ¹⁴⁹Pm, ¹⁷⁷Lu, ⁴⁷Sc, ¹⁴²Pr, ¹⁵⁹Gd, ²¹²Bi.

A second class of preferred compounds includes those in which F is a chelant residue of formula (I)



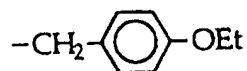
or of formula (II)

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in which

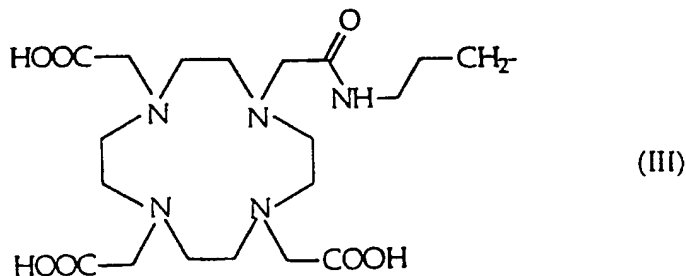
- A corresponds to a simple bond or to a $-(CH_2)_sR_1-$ group where s is an integer from 1 to 5 and
- 10 R_1 is a simple bond or is equal to CONH, NHCO, NHCSNH, $C_6H_4NHCSNH$, COO, OCO, O, S,
- B corresponds to $-D-(CH_2)_t-$, in which
- t is an integer from 1 to 9 and
- D is a simple bond, or is equal to $-O-$, $-NH-$,
- 15 R in each compound can be H or CH_3 or CH_2-O-Bz or



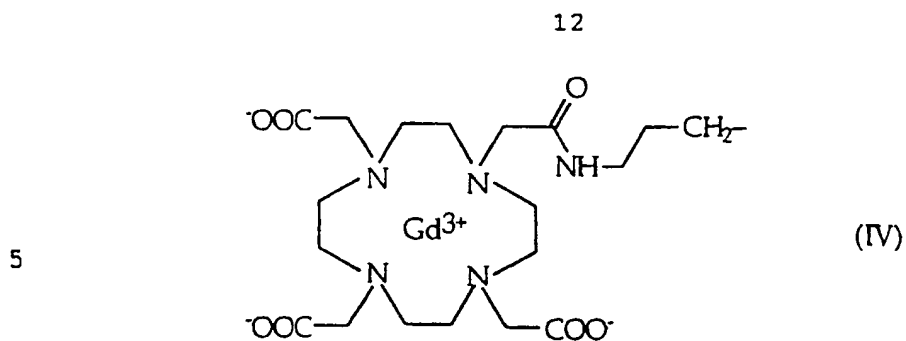
or any combination of them,

as well as their metal chelates or their salts.

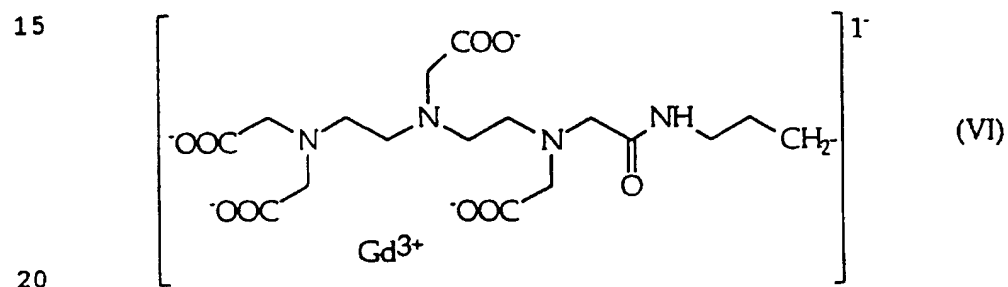
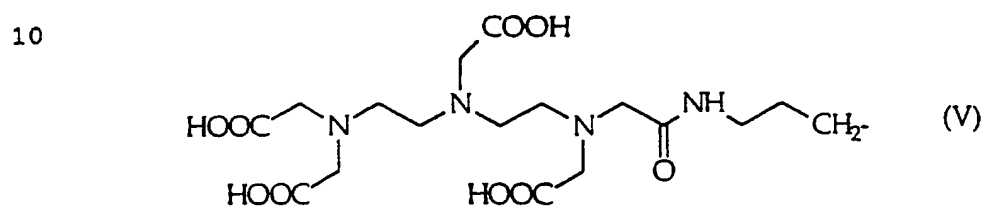
- 20 A third class of preferred compounds is that in which F is a residue of formula (III) or (IV)



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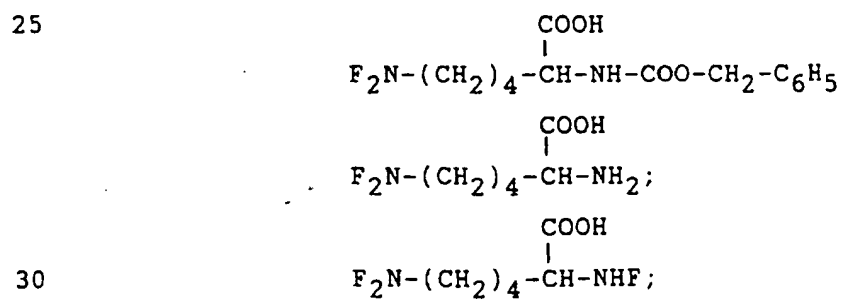


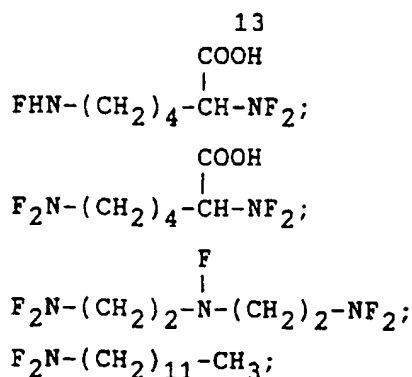
or of formula (V) or (VI)



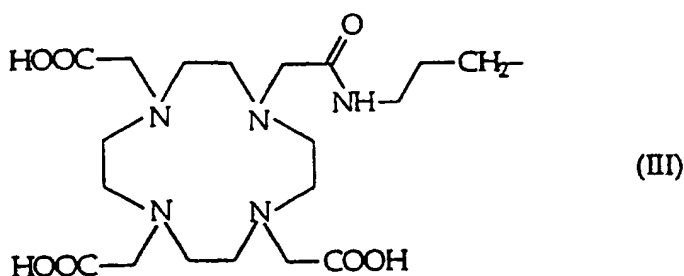
or one of their salts.

A fourth class of preferred compounds includes the following:

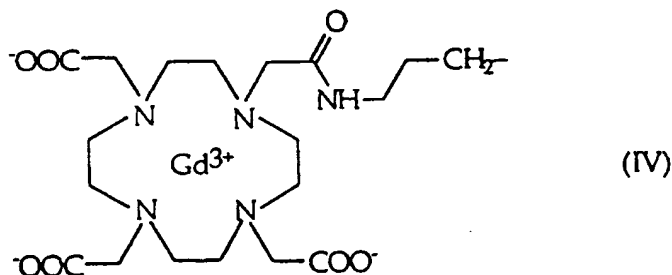




where F is a chelant residue of formula (III),



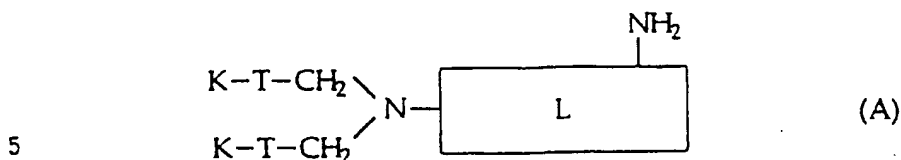
or a chelate of formula (IV),



or a salt thereof. Other preferred compounds are those in which F is a residue of formula (III) or (IV) and L is selected from the group constituted by [Lys³]-bombesin, insulin, myoglobin, albumin, cytochrome c, chymotrypsinogen A, polylysine.

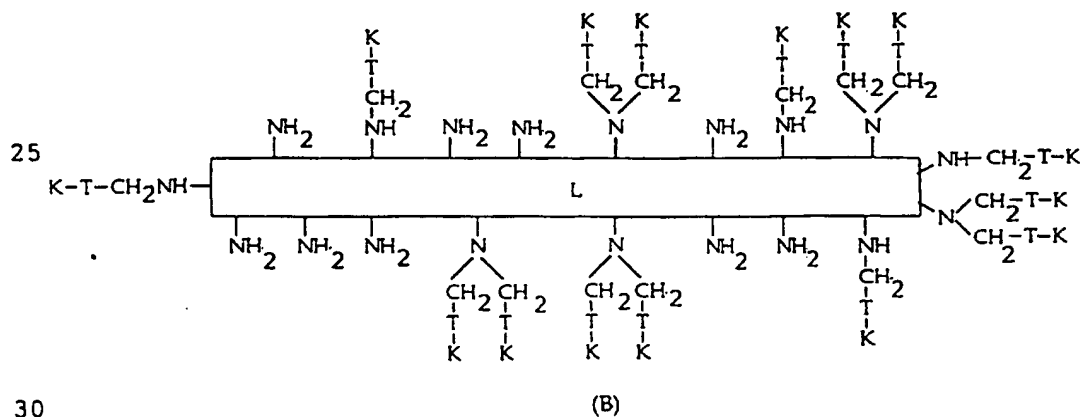
To clarify the structure of the compounds of the invention, as an example, in formula (A) below the structure of a polychelant/polychelate derivative,

object of the present invention is schematised



where L, K, T, are defined as above. The product schematised in formula (A) was obtained starting from an organic backbone L containing 2 primary amino groups ($m = 2$), one of which was dialkylated with two K-T-CH₂-residues. The resulting compound therefore possesses two chelant/chelate moieties on a nitrogen ($n = 2$) and a free amino group ($p = 1$). Since $m-p$ is equal to 1, therefore $n > m-p$. Consequently $\rho = 2$. A product of this type is described in Example 4.

Analogously, in formula (B) below a structure is schematised of a polychelant/polychelate derivative, object of the present invention obtained starting from a polyaminic organic backbone L containing 19 primary amino groups ($m = 19$).



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Said backbone L was alkylated with 15 K-T-CH₂-residues. The resulting compound possesses 9 free amino groups ($p = 9$) and a total of 15 alkylene groups ($n = 15$). Since $m-p$ is equal to 10, therefore $n > m-p$ and
5 $p = 1.5$, that is > 1 . A product of this type is described in Example 10.

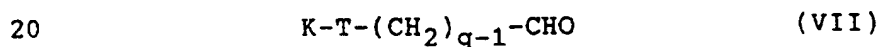
The compounds of the present invention can, for example, be obtained by any of the synthetic processes known to experts for alkylation of primary amines such
10 as alkylation with alkyl halides. Nevertheless, we have also surprisingly found that, in contrast to the prevailing teaching of the state of the art, also reductive alkylation is a useful method and is particularly efficient for the preparation of the
15 tertiary amines that are object of the present invention. Indeed, a vast quantity of literature references exists (G.E. Meares & R.E. Feeney, Anal. Biochem. 224, 1-16, 1995) in which the use of reductive alkylation is described for the conjugation of different
20 aldehydes with proteins in the presence of suitable reducing agents. In general however, with the sole exception of formaldehyde, the formation of tertiary amines is not observed. This is attributed to the reciprocal steric hindrance between the amine and the
25 aldehyde. Recently, it has been reported that with a less sterically hindered amine such as glycine the formation of the tertiary amine can occur, but only in a very low percentage, as a by-product of the reaction (J.-P. Sani et al., Tetrahedron Lett. 35, 1181-1184,
30 1994). Even in a very recent article, where the reductive alkylation of proteins by aldehyde derivatives

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of chelating agents was described, no disclosure of double alkylation was reported, in spite of the careful optimisation of various experimental parameters (V.V. Somayaji et al., Appl. Radiat. Isot. 47(1), 71-77, 1996). On the contrary, we have surprisingly found that under particular experimental conditions the steric hindrance of the two reagents is no longer a limiting factor. Namely tertiary amines can be obtained in good yield even when large aldehydes are taken as the starting material and the primary amino groups belong even to macromolecules.

In general, it is essential that the aldehyde, linked to the chelant residue, or to one of its chelates, or salts, by means of an aliphatic chain as described above, is in molar excess of from 3 to 40 times the number of primary amino groups.

In particular, the procedure according to the present invention includes the reaction of a chelant compound of formula (VII)



where K, T, q are as described above, or one of its chelates or salts, with a polyamino compound of formula (VIII)



where L and m are as described above, in a reaction medium, under conditions of reductive amination, characterised by the fact that: i) the compound of formula (VII) is in a 3 to 40 fold molar excess with respect to the m primary amino groups, ii) the reaction is carried out in the presence of a reducing agent specific for the imine bond, but not for the aldehyde,

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said reducing agent being in a 3-60 fold excess with respect to the starting primary amino groups.

Preferably, said reaction medium is selected from the following: an aqueous buffer, of pH from 5 to 10, a
5 low molecular weight alcohol, an aprotic dipolar solvent, or even a mixture thereof, the temperature of reaction is between -5 and 60°C for a length of time of between 2 and 170 h.

A first and particularly preferred realisation of
10 the procedure of the present invention envisages:

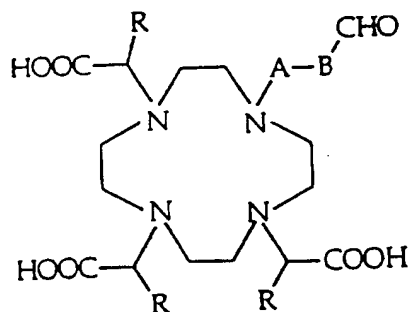
- the use of a metal chelate compound of formula (VII), or one of its salts, in a molar excess of about 10-35 fold with respect to the total number of primary amino groups;
- 15 - the reaction medium is an aqueous buffer at a pH of 7-9, or methanol, or a mixture of the two;
- the reducing agent is sodium cyanoborohydride;
- the temperature varies between 15-30°C;
- the reaction time is between 10-72 h.

20 In a second, particularly preferred realisation of the procedure of the present invention the reductive alkylation reaction occurs between a chelant of formula (VII) and a polyamino compound of formula (VIII) as above described, followed by the subsequent formation of
25 the relative metal complex and/or one of its salts.

Intermediate aldehydes preferred for the preparation of the compounds of the present invention are compounds of formulae (Ia) and (IIa) below:

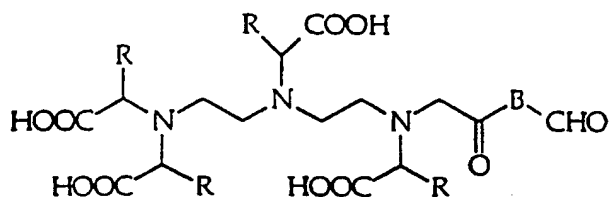
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(Ia)

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(IIa)

15

in which

A corresponds to a simple bond or to a $-(CH_2)_sR_1-$ group where s is an integer from 1 to 5 and R_1 is a simple bond or is equal to CONH, NHCO, NHCSNH, $C_6H_4NHCSNH$, COO, OCO, O, S,

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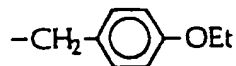
B corresponds to $-D-(CH_2)_t-$, in which

t is an integer from 1 to 9 and

D is a simple bond, or is equal to $-O-$, $-NH-$,

R is H or $-CH_2-O-Bz$ or

25



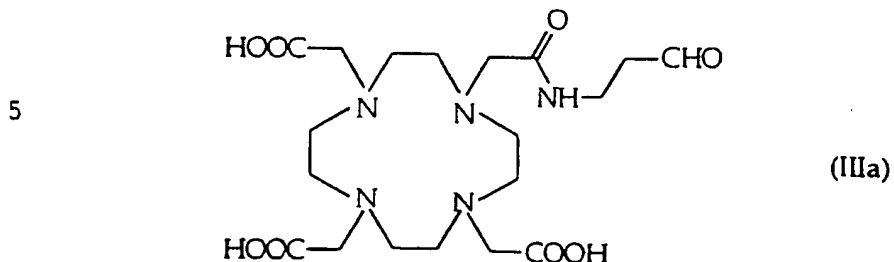
with the condition that one only of the R substituents can be different from H,

being said compounds (Ia) and (IIa) either as a chelant or as a complex with a bi- or trivalent metal ion selected from those described previously, or one of their salts.

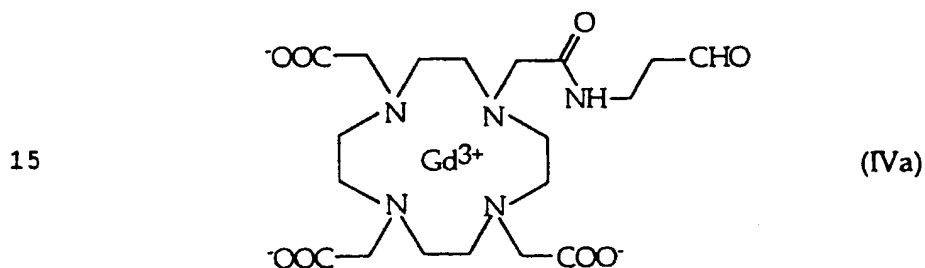
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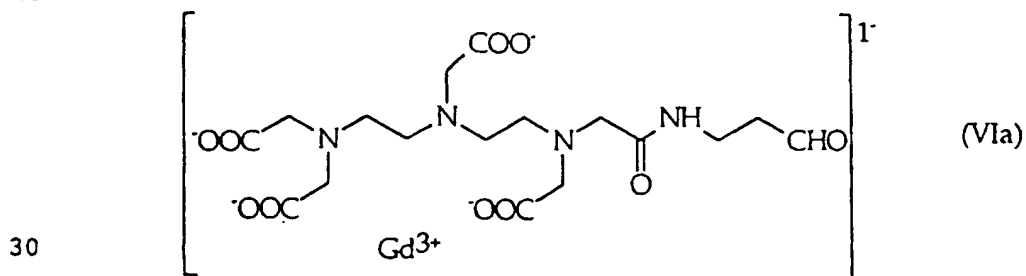
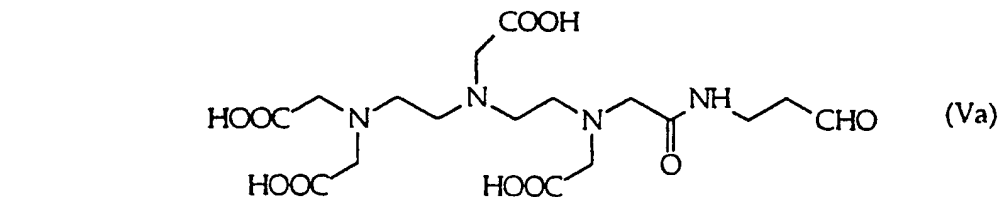
Particularly preferred are the following intermediates of formula from (IIIa) to (VIa)



10- $[2\text{-oxo-2-}[(3\text{-oxopropyl})\text{amino}]\text{ethyl}]\text{-1,4,7,10-tetraazacyclododecan-1,4,7-triacetic acid}$, and its relative gadolinium complex of formula (IVa)



3,6,9,12-tetraaza-11,15-dioxo-3,6,9-tris(carboxymethyl)-pentadecanoic acid of formula (Va), and its relative gadolinium complex of formula (VIa)



and their salts.

Particularly preferred carriers on which to insert the chelants of formula (Ia), (IIa), or their metal complexes, are amino derivatives such as primary amines
5 and polyamines, amino acids, polypeptides, polyamino acids, proteins, antibodies, amino sugars and linear or branched polymers containing primary amino groups or their derivatives.

Particularly preferred amines are for example
10 spermidine, spermine, ethanolamine, phosphatidyl-ethanolamine and its derivatives, sphingosine, diethylenetriamine, alkylamines, alkylenediamines, tris-(2-aminoethyl)amine, jeffamine, N-glucosamine.

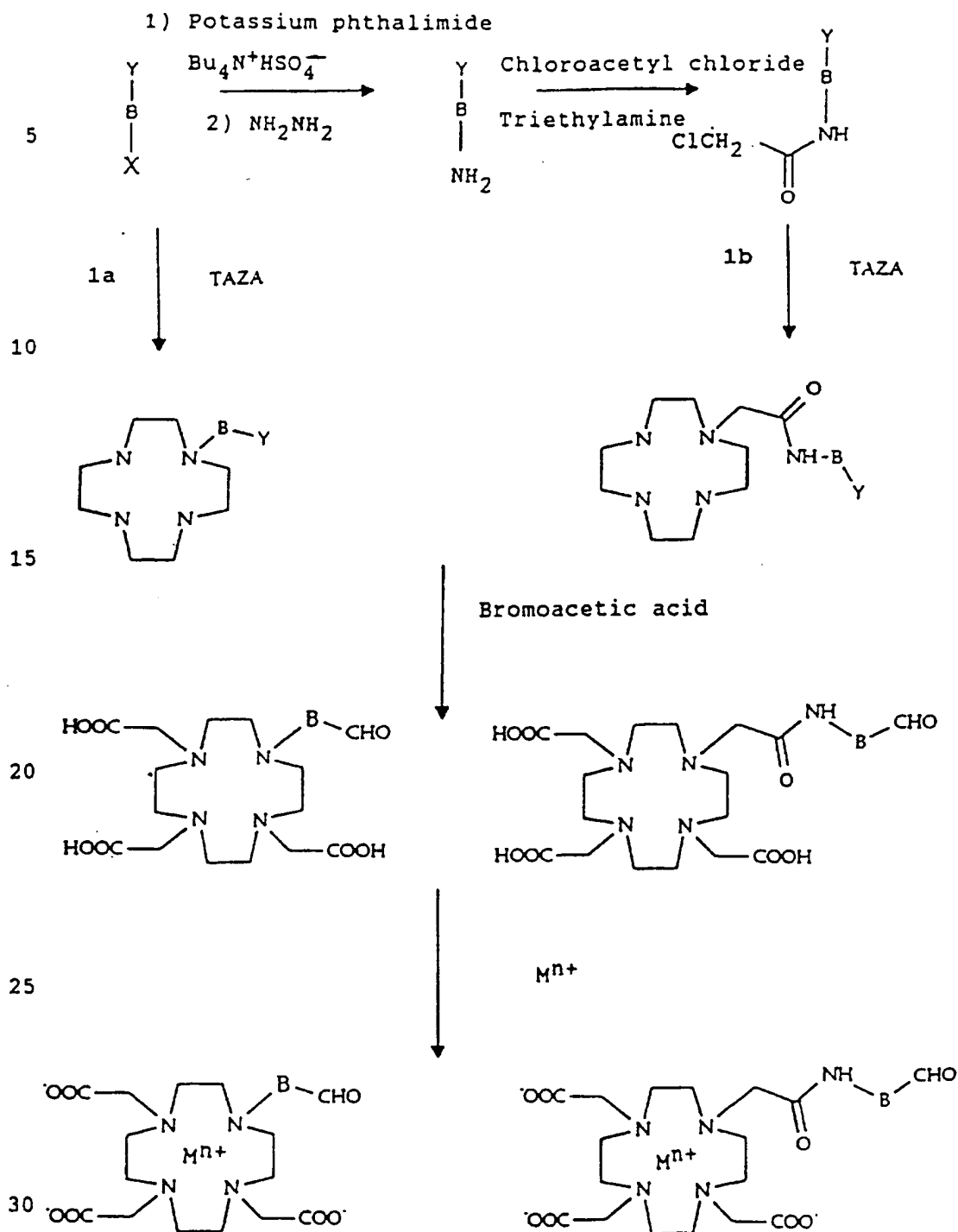
Particularly preferred amino acids are lysine,
15 ornithine, glycine, 4-aminobutyric acid, aminocaproic acid, taurine.

Particularly preferred macromolecules are [Lys³]-bombesin, insulin, chymotrypsinogen A, myoglobin, albumin, cytochrome c, branched and linear polylysine,
20 ramified and linear polyornithine, hormones, growth factors.

Particularly preferred are those products obtained by reaction between compounds of formula (IVa) and (VIa) with the amino derivatives defined previously.

25 The preparation of the preferred intermediates of the invention is outlined in the following schemes 1 and 2, for the cases in which the R groups are equal to H in the compounds of formula (Ia) and (IIa) while group A corresponds to -CH₂CONH- (via 1b) and, respectively, to
30 a simple bond (via 1a) and B is as above defined for formulas (Ia) and (IIa).

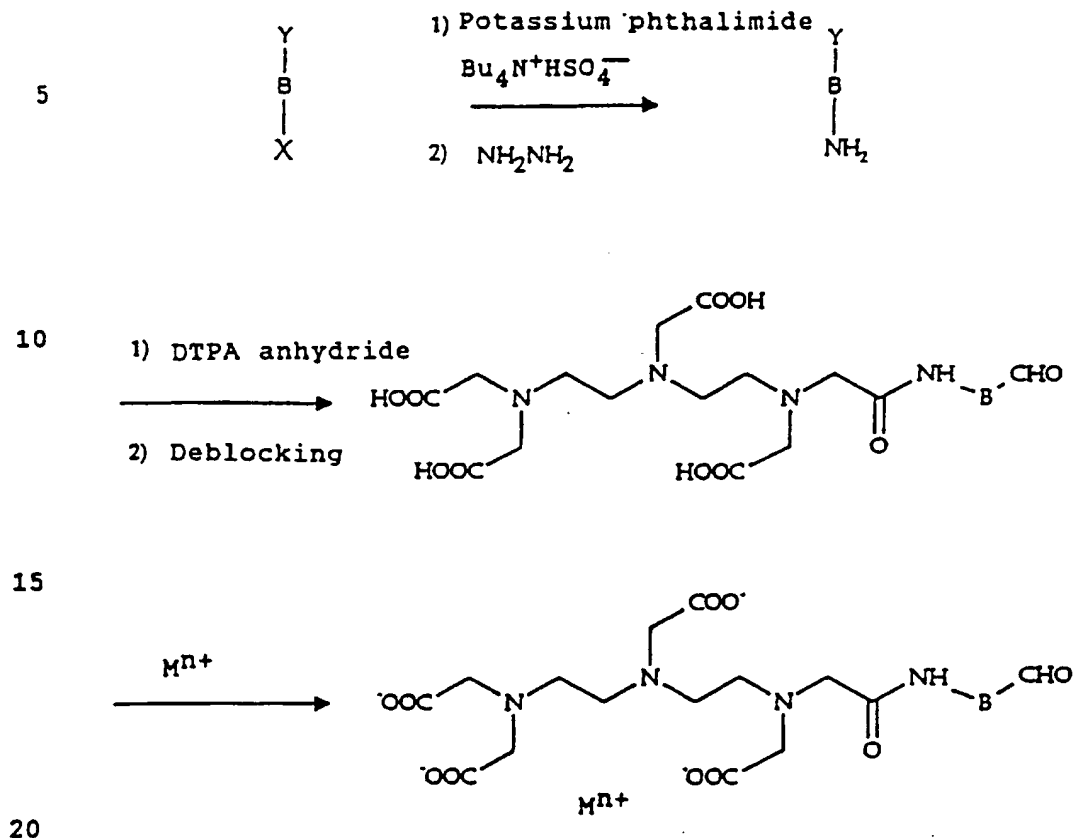
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Scheme 1



The process illustrated in Scheme 1 can be summarised as follows:

- preparation of a masked aldehyde building block, corresponding to the formula X-A-B-Y, where A can be absent (scheme 1a, A = simple bond) or present (scheme 1b, A = CH₂CONH), X represents a leaving group, preferably selected from the group constituted by halogens, OTs, OMs, OTf and Y represents the aldehyde protected with a protective group preferably selected from those that are labile at acidic pH, in particular the derivatives of 1,3-dioxolane and 1,3-dioxane;
- reaction between TAZA (1,4,7,10-tetraazacyclododecane) and the aldehyde building block prepared previously to give a 1:1 alkylation product;
- reaction with bromoacetic acid and simultaneous deblocking of the protected aldehyde to give the chelant agents of formula (Ia), in which R = H.
- possible formation of the desired metal complex and/or of the salts thereof by means of chelation of the metal ion, preferably carried out by reacting the chelant agents of formula (Ia) with a metal, either in its salt or oxide form, possibly in the presence of the quantity of base or acid necessary for neutralisation to give the relative metal complexes.

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Scheme 2



The process illustrated in Scheme 2 can be summarised as follows:

- 25 - preparation of a masked aldehyde building block, corresponding to the formula X-B-Y, where X represents a leaving group, preferably selected from the group constituted by halogens, OTs, OMs, OTf and Y represents the aldehyde protected with a
- 30 protective group preferably selected from those that are labile at acidic pH, in particular the

derivatives of 1,3-dioxolane and 1,3-dioxane and B is as previously defined;

- transformation of X into an amine to give the carbonyl-protected $H_2N-B-CHO$ -block;
- 5 - condensation between the commercially available dianhydride of DTPA (diethylenetriaminepentaacetic acid) with the aldehyde block prepared previously;
- deblocking of the protected aldehyde to give the chelant agents of formula (IIa), in which $R = H$;
- 10 - possible formation of complexes and their salts as described for Scheme 1.

Unlike the generalised teaching of the state of the art, it has surprisingly been found not only possible, but indeed convenient, to conjugate directly and with
15 high yield the amino or polyamino carriers cited above with metal complexes of the chelants of formula (Ia) and (IIa) rather than with the respective chelants. This is particularly advantageous because the products obtained do not require a subsequent complexation step. In this
20 way, an incomplete and/or non-specific incorporation of the metal ions into the complex is avoided. Furthermore, potentially degradable products need not to be subjected to the often very drastic conditions required for complexation. Finally, the purification of the final
25 product is much more straightforward to perform.

The reductive alkylation reaction for the preparation of the polychelants/polychelates, objects of the present invention, occurs according to the following indications:

- 30 - reaction of a chelant of formula (Ia) or (IIa) or of one of their metal complexes with the amino

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carrier of interest or one of its salts in a 3 to 40 fold excess with respect to the total number of primary amino groups of the same, preferably 10-35 fold;

- 5 - the reaction medium is generally an aqueous buffer such as phosphate, borate, bicarbonate, carbonate, acetate or similar at a pH from 5 to 10; or is constituted by an alcohol of low molecular weight such as, for example, methanol, ethanol, propanols, butanols; or by an aprotic dipolar solvent such as, for example, DMF, DMSO, DMA; a mixture with aqueous buffer solutions such as those mentioned above is also possible and sometimes preferred;
- 10 - the reaction occurs in the presence of a reducing agent specific for the imine bond, but not for the aldehyde, in a 3 - 60 fold excess with respect to the amino groups of the starting amino carrier, whereas preferred examples of said reducing agent are represented by sodium cyanoborohydride (NaCNBH₃), pyridine borane, trimethylamine borane and analogues;
- 15 - the temperature of the reaction is between -5 and 60°C, but preferably between 15-30°C;
- 20 - the reaction time varies from 2 to 170 h, but preferably from 10 to 72 h.
- 25

Particularly preferred buffers are those of phosphate and borate at pH values between 7 and 9. Preferred solvents are methanol, DMF and DMSO. The concentration of the amino carrier in the reaction medium is between 0.1 and 40% (w/v). The preferred reducing agent is sodium cyanoborohydride.

30

Obviously, when the reductive alkylation reaction is carried out starting from the chelant of formula (Ia) or (IIa) with the amino carrier, the subsequent step involves the formation of the relative metal complex, according to known methods and techniques.

A further unexpected advantage of reductive alkylation, as opposed to other methods of alkylation, concerns the specificity of the reaction which permits its application to proteins and amino derivatives that contain other reactive groups. The specificity of the reaction has, for example, been confirmed with proteins. In all cases, analysis of the amino acids of the modified proteins, has been shown to be identical to that of the starting protein except for the lysine residue and possibly the amino acid at the amino terminal. The possibility that compounds of formula (Ia) and (IIa) or their metal complexes may react with side chains of other amino acids to give hydrolysable products, which could escape identification during analysis of the amino acids, has been excluded.

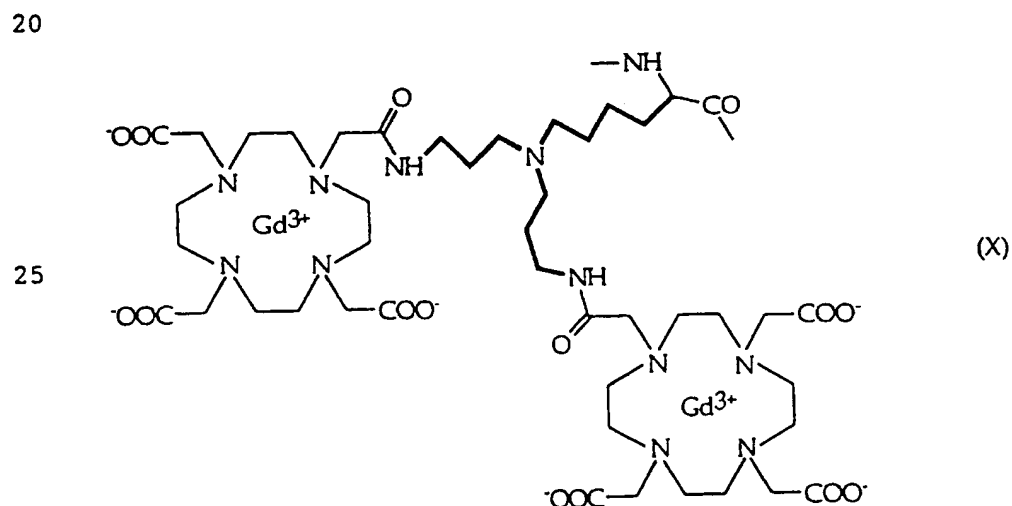
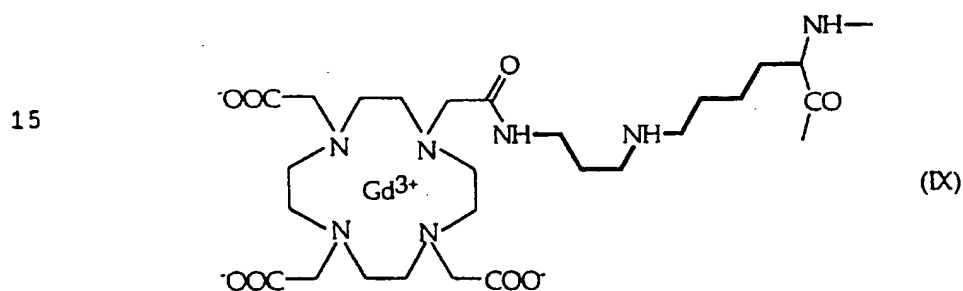
Indeed, the gadolinium complex of formula (IVa), for example, did not react with a model peptide devoid of free primary amino groups. This experiment was conducted with the luteinizing hormone-releasing hormone, LHRH, a peptide with the amino-terminal group blocked, having the sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-amide and molecular mass 1182.3. Mass spectrometry has shown that its mass remains unchanged after reaction with the gadolinium complex of formula (IVa) under the conditions adopted for the modification of proteins.

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On the contrary, the products that are obtained by reaction of the gadolinium complex of formula (IVa) with the proteins selected for the present invention contain a number of complex residues that is greater than the number of amino groups theoretically available (α and ϵ).

Consequently, a large proportion of the (α and ϵ)-amino groups have undergone double alkylation.

For example, reaction of the lysine ϵ -amino groups with gadolinium complex of formula (IVa) gives the modified lysine residues of formula (IX) and (X):



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Hydrolysis of such modified residues gives N^{ϵ} -(3-aminopropyl)lysine and $N^{\epsilon},N^{\epsilon}$ -bis(3-aminopropyl)lysine which can be utilised in the characterisation of the type of substitution.

- 5 As an additional exemplification, Table 1 below illustrates the peculiar advantages of the present invention, in which the products obtained are characterised in terms of the parameter which is always greater than 1.

Table 1. Comparison of the characteristics of some preferred products of the invention and the state of the art [P=Formula (IV)].

| | Conjugated product | m | p | n | Gd (%w/w) | Reference |
|----|------------------------------------|-----|-------|------|-----------|---|
| 5 | (Gd-DTPA) _n HSA | 60 | 25 | 35 | 1 | 6.5% Vexler, V.S. et al., <i>Invest. Radiol.</i> 1992, 27, 935-941. |
| | (Gd-DTPA) _n BSA | 60 | 41 | 19 | 1 | 3.3% Ogan M. et al., <i>Invest. Radiol.</i> , 1987, 22, 665-671 |
| 10 | (DOTA) _n cytochrome c | 19 | 10.21 | 8.79 | 1 | 8.1% Lewis, M.R. et al., <i>Bioconj. Chem.</i> 1994, 5, 565-576 |
| | (Gd-DTPA) _n PL | 156 | 116 | 40 | 1 | 15.7% Berthezène, Y. et al., <i>Invest. Radiol.</i> 1992, 27, 346-351 |
| | F ₂ -Lys(Z) | 1 | 0 | 2 | 2 | 21.3% The present invention Example 3 |
| | F ₂ -Lys | 2 | 1 | 2 | 2 | 23.4% The present invention Example 4 |
| 15 | F ₃ -Lys | 2 | 0 | 3 | 1.5 | 24.3% The present invention Example 5 |
| | F ₄ -Lys | 2 | 0 | 4 | 2 | 24.8% The present invention Example 6 |
| | F ₅ -diethylenetriamine | 2 | 0 | 4 | 2 | 25.4% The present invention Example 8 |
| | F ₂ -dodecylamine | 1 | 0 | 2 | 2 | 22.8% The present invention Example 9 |

(continued)

(continued)

| | | | | | | | | |
|---|------------------------------------|----|-----|------|------|-------|-----------------------|------------|
| 5 | F_2 -[Lys ³]bombesin | 1 | 0 | 2 | 2 | 11.3% | The present invention | Example 10 |
| | F_n -insulin | 3 | 0.1 | 4.6 | 1.59 | 8.5% | The present invention | Example 11 |
| | F_n -myoglobin | 20 | 0.5 | 27.9 | 1.43 | 13.5% | The present invention | Example 12 |
| | F_n -chymotrypsinogen A | 15 | 0.2 | 28.2 | 1.91 | 10% | The present invention | Example 13 |
| | F_n -cytochrome c | 19 | 9 | 14.6 | 1.46 | 10.9% | The present invention | Example 14 |

The table clearly shows that the products obtained according to the teaching of the state of the art are characterised by $\rho = 1$, while those of the present invention have $\rho > 1$ (in some cases up to the maximum value possible, i.e. 2, which means that the complete disubstitution of the primary amino groups of the carrier was obtained). Furthermore the percent content (by weight) of Gd per carrier molecule, is on average much higher for the compounds of the present invention, when the same carriers are compared. Indeed, this value is, for example, equal to 8.1% for the (DOTA)_{8.79}cytochrome c product described by Lewis et al. (Bioconj. Chem. 1994, 5, 565-576), assuming complete complexation, while for the derivative of cytochrome c described in Example 11 of the present invention this value is 10.9%. Assuming, for an MRI experiment, the administration of a dose of 100 μmol of Gd/kg, in the case of the product of Lewis et al. a total of 11.4 μmol of product/kg should be administered, while for the product of the present invention only 6.8 $\mu\text{mol/kg}$ (that is, almost half) is required. Actually the same authors (Lewis et al.) sustain that the efficiency of the chelation with metal ions varies from 84.5 to 97.7% (Bioconj. Chem. 1994,5,565-576, Table 1 pg. 567). Considering that for the product of the present invention this value is 100%, the final benefits are clearly even greater.

The compounds, objects of the present invention, have a wide field of application. In particular, the complexes with paramagnetic metals can be used, when suitably formulated, in all the diagnostic procedures

based on magnetic resonance.

The chelates, objects of the present invention, can also be used in nuclear medicine. In this case however the metal ion that is chelated is a radioisotope that emits particles for example ^{51}Cr , ^{67}Ga , ^{68}Ga , ^{111}In , $^{99\text{m}}\text{Tc}$, ^{140}La , ^{175}Yb , ^{153}Sm , ^{166}Ho , ^{90}Y , ^{149}Pm , ^{177}Lu , ^{47}Sc , ^{142}Pr , ^{159}Gd , ^{212}Bi .

The metal complexes, objects of the present invention, can also be encapsulated in liposomes, employed as single or multilamellar vesicles or used in association with an anionic, hydrophilic and water-soluble "carrier". This "carrier" can be a saccharide, an oligosaccharide, a polysaccharide or a glycosaminoglycan containing sulphate groups, such as for example heparin sulphate, chondroitin sulphate, dermatan sulphate.

In the experimental part the methods of preparation of a few compounds of the present invention are reported.

EXAMPLE 1 - Methods

Analytical methods for the characterisation of the prepared compounds.

Mass spectrometric analysis

The mass value of products of the present invention have been determined using the technique of Electrospray Ionisation (ESI-MS), which permits the analysis of metal complexes. The macromolecular type products were also analysed using the technique termed Matrix Assisted Laser Desorption Ionisation Time Of Flight (MALDI-TOF) with a Lasermat 2000 (Finnigan Mat) instrument and α -ciano-4-hydroxycinnamic acid (ACH) or sinapinic acid as

the matrix.

Elemental analysis

The percentages of C, H, and N were obtained according to standard methods. The gadolinium content
5 was determined by means of emission spectrometry (ICP-ES) or x-ray fluorescence (XRF). The samples were completely mineralised in a microwave apparatus prior to the analysis.

Analysis of amino acids

10 In the case of compounds obtained starting from proteins, the protein content was determined by means of quantitative analysis of the amino acids. The analyses were conducted after hydrolysis of the products at 110°C in 6 N HCl for 20-48 h. The hydrolysates were analysed
15 on a Carlo Erba 3A-29 analyser, equipped with a ninhydrin post-column detector.

Determination of free amino groups

For the macromolecular type products, the "p" parameter (remaining free amino groups after conjugation) has been
20 determined using an assay based on fluorescamine as described in Stocks S.J., Andrew J.M., Ramey C.W., Brooks D.E., Anal. Biochem., 1986, 154(1), 232-234. The response for the compound under analysis has been compared with that of the corresponding non-modified
25 macromolecule. In the case of compounds obtained starting from proteins, the protein content was determined by means of quantitative analysis of the amino acids. The concentrations of non-modified proteins were determined on the basis of the absorption
30 coefficient in the case of insulin ($\epsilon_{277.5 \text{ nm}} = 0.957 \text{ mL} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$) and chymotrypsinogen A ($\epsilon_{282 \text{ nm}} = 2.03$

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mL·mg⁻¹·cm⁻¹), while for myoglobin and cytochrome c the pyridine-hemochromogen method was used (Riggs, A., Methods in Enzymol., 1981, 76, 20-21).

Size exclusion chromatography

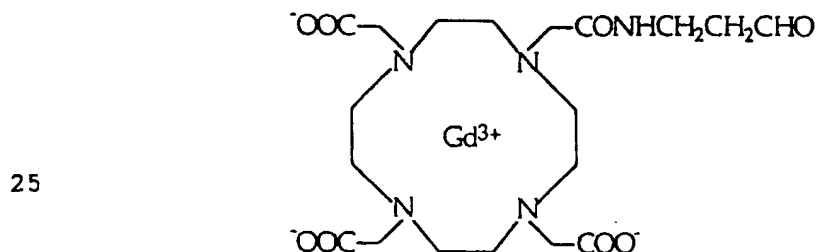
5 In the case of compounds obtained starting from proteins, the homogeneity of the product has been verified by size exclusion chromatography. Samples of each compound were injected (25 µL) onto a Superdex 75-HR 10/30 column (Pharmacia) equilibrated in 0.2 M
10 NH₄HCO₃. Chromatography was carried out in a cold room (6-7°C) at 0.5 mL/min and followed spectrophotometrically at 280 nm.

Relaxivity measurement

For some Gd-containing products the relaxivity r₁
15 was measured using a Bruker Minispec 120, at 0.5 T and 39°C.

EXAMPLE 2

Gadolinium complex of 10-[2-oxo-2-[(3-oxopropyl)amino]ethyl]-1,4,7,10-tetraazacyclododecan-1,4,7-triacetic acid
20 acid



A) 10-[2-oxo-2-[(3-oxopropyl)amino]ethyl]-1,4,7,10-tetraazacyclododecan-1,4,7-triacetic acid (Compound of formula IIIa)
30

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The product was prepared according to the procedure described in Example 4 of patent application WO 95/32741, page 49.

The ^1H -NMR, ^{13}C -NMR, IR and MS spectra are in agreement with the structure indicated.

B) Gadolinium complex of 10-[2-oxo-2-[(3-oxopropyl)-amino]ethyl]-1,4,7,10-tetraazacyclododecan-1,4,7-triacetic acid (Compound of formula IVa)

Gadolinium oxide (1.14 g; 0.00315 mol) is added to a solution of compound A) (2.9 g; 0.0063 mol) in H_2O (3000 mL). The reaction mixture is heated to 50°C for 20 h and the reaction followed by HPLC. The reaction mixture is filtered through a Millipore (0.45 μm) filter. After evaporating to dryness, the desired product is obtained (3.85 g; 0.00627 mol).

Quantitative yield m.p.: $> 280^\circ\text{C}$

Quantification of ethylene glycol (GC): 1.35% (external standard)

HPLC titre: 94% (in area %)

Titre K.F.: 9.25%

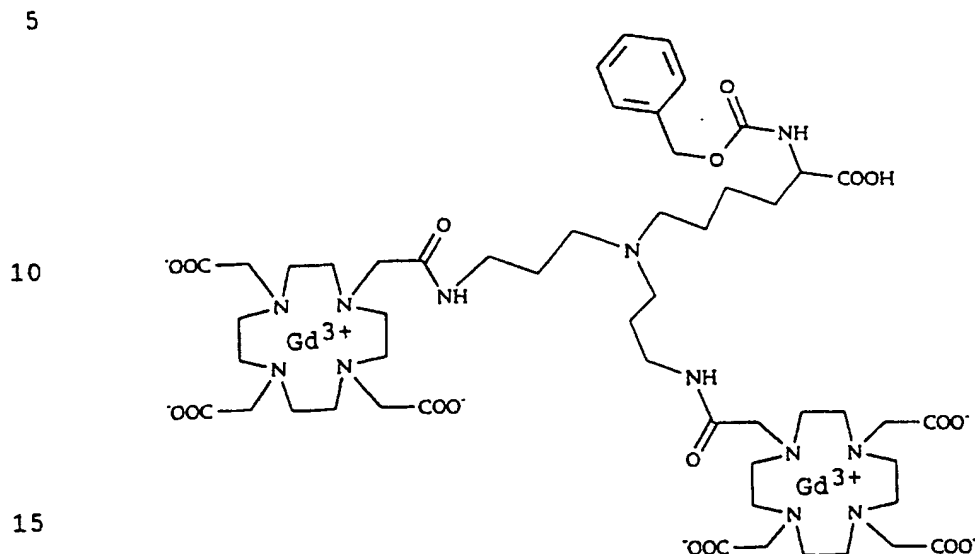
Elemental analysis for $\text{C}_{19}\text{H}_{30}\text{GdN}_5\text{O}_8 \cdot 3.18 \text{ H}_2\text{O}$

| | C | H | Gd | N |
|------------|-------|------|-------|-------|
| calculated | 33.74 | 5.51 | 23.25 | 10.36 |
| Found | 33.36 | 5.67 | 23.30 | 10.16 |

The IR and MS spectra are in agreement with the indicated structure.

EXAMPLE 3

Gadolinium complex of N^α-carbobenzyloxy-N^α,N^α-bis-[4-
aza-5-oxo-6-(1,4,7,10-tetraazacyclododecyl-4,7,10-tri-
acetate)hexyl]-L-lysine



4 g of compound (IVa) obtained as described in Example 2B (6.5 mmol) are dissolved in 10 mL of methanol and cooled to -5°C , in a nitrogen atmosphere. Separately, a solution of 0.45 g of N^α-carbobenzyloxy-L-lysine (Z-lysine, 1.6 mmol) and 0.1 g KOH is prepared in 4 mL of MeOH. This is added to the solution of compound (IVa) obtained as described in Example 2B in the flask. Finally, 0.6 g of NaCNBH₃ (9.5 mmol) are added. The mixture is then stirred constantly at -5°C for 18 h. The solvent is then evaporated and the residue taken up with 10 mL of a mixture of CH₃CN/H₂O/trifluoroacetic acid (TFA) 10:90:0.1. This is then filtered to eliminate the insoluble residue and purified on a LiChrosorb RP-18 25x250 mm column (E. Merck). The pure compound is eluted

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isocratically with the same $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{TFA}$ mixture. The homogeneous fractions are combined on the basis of an analysis carried out under the same conditions on the analytical column and then evaporated to dryness. The residue is washed with ether and dried under vacuum. Approximately 1.9 g of the desired product is obtained containing traces of TFA.

K.F. titre: 3.43% (w/w)

HPLC titre: 98% (in area %)

Column: E. Merck LiChrospher 100 RP-18 (5mm); 250x4 mm

Eluent A: 5 mM phosphate pH 3

Eluent B: CH_3CN

10 min 10%B; gradient from 10 to 30% B in 10 min.

Flow rate: 1 mL/min

| | | | | |
|--------------------|-------|------|------|-------|
| Elemental analysis | C | H | N | Gd |
| % calculated: | 33.03 | 3.97 | 6.80 | 12.72 |
| % found: | 33.22 | 3.88 | 7.28 | 12.15 |

ESI-MS spectrum: 1476 (MH^+)

To demonstrate the structure of the product, it is hydrolysed in 6 N HCl for 20 h at 110°C. The hydrolysis product is $\text{N}^\epsilon, \text{N}^\epsilon$ -bis(3-aminopropyl)-lysine as confirmed by mass spectrometry (MH^+ : 261) and NMR spectrometry. During chromatography on the LiChrosorb RP-18 (25x250 mm) column, fractions containing the monoalkylation product are also collected.

Yield: 14% (200 mg)

ESI-MS spectrum: 879 (MH^+)

The ρ ratio for the product described in this example is 2.

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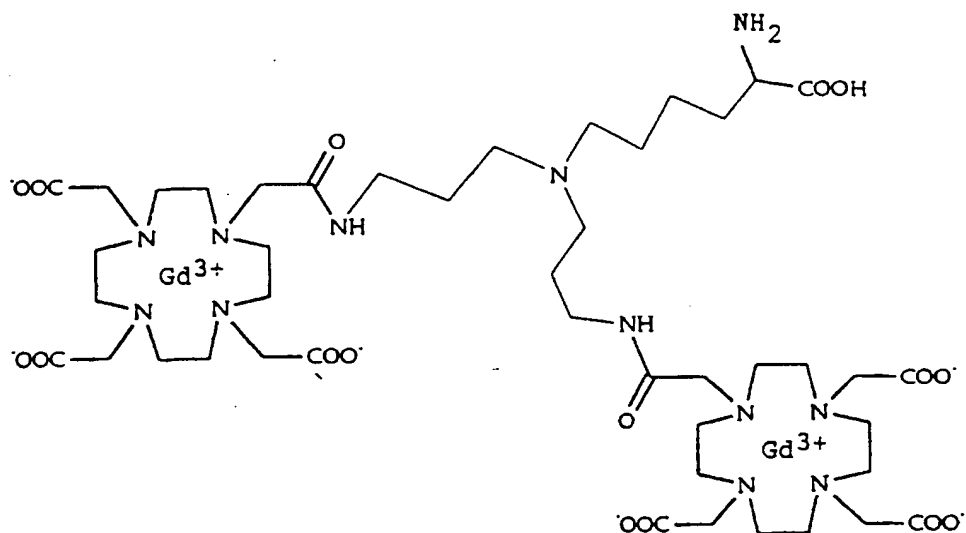
EXAMPLE 4

Gadolinium complex of N^E,N^E-bis-[4-aza-5-oxo-6-(1,4,7,10-tetraazacyclododecyl-4,7,10-triacetate)hexyl]-L-lysine

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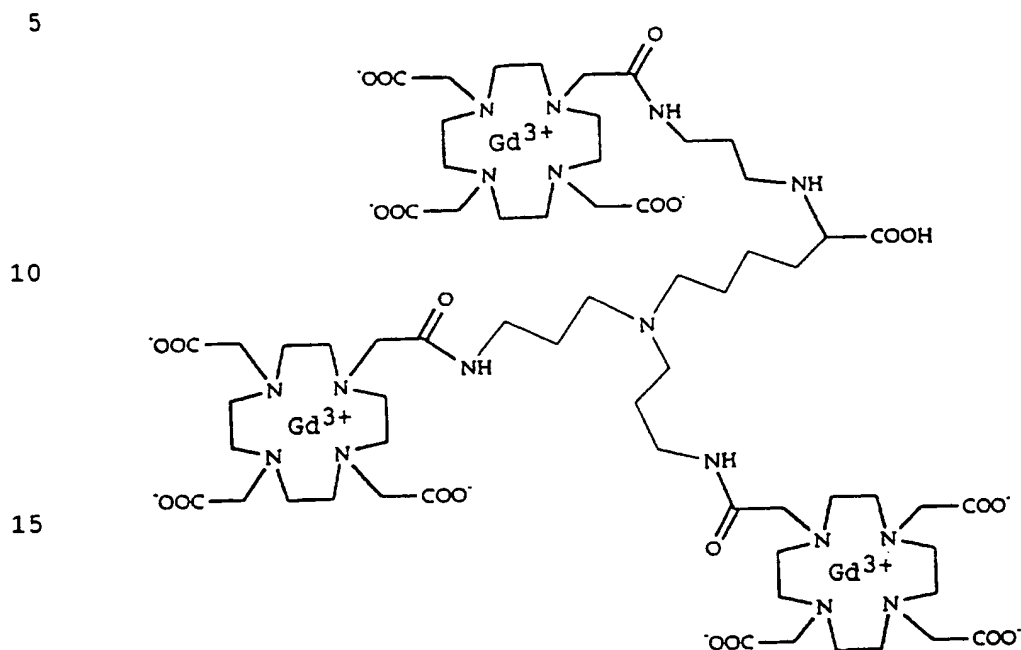
0.5 g of the compound obtained in Example 3 are dissolved in 50 mL of methanol, to which is added 0.25 g of 20% Pd(OH)₂/C. This is then hydrogenated at ambient P and T for 10 min, after which the flask is washed with nitrogen to remove the CO₂ formed. Hydrogenation is then continued for another 10 min. The reaction mixture is then filtered and evaporated. The residue is suspended in ethyl ether, filtered and dried. Yield 400 mg (88%)

ESI-MS spectrum: 1342 (MH⁺)

The ρ ratio for the product described in this example is 2.

EXAMPLE 5

Gadolinium complex of $\text{Na}^a \cdot \text{NE} \cdot \text{NE}^{(a)}$ -tris-[4-aza-5-oxo-6-(1,4,7,10-tetraazacyclododecyl)-4,7,10-triacetate)hexyl]-L-lysine



2 g of compound (IVa) obtained as described in Example 2B (3.25 mmol) are dissolved in 10 mL methanol and cooled to -5°C. A solution of 100 mg lysine·HCl (0.55 mmol) and 26 mg LiOH (1.1 mmol) in 4 mL methanol and, finally, 310 mg of NaCNBH₃ (4.93 mmol) are then added to this. The reaction mixture is stirred at -5°C for 60 h, at which point the solvent is evaporated, and the residue taken up in 5 mL H₂O. This is filtered and purified on an ion exchange resin (AG-50W-X4, Bio-Rad) equilibrated in 0.1 M pyridine/acetic acid pH 5.6. After removing excess product (IVa) and by-products of the reaction from the column, the product of interest is eluted with 1 M pyridine/acetic acid pH 5.6. The

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fractions containing product (TLC: Silica 60-F254, Eluent: ethanol/25% ammonia 1:1; $R_f=0.36$) are combined, dried, taken up in water and lyophilised extensively. 425 mg of product are obtained (40% of the lysine).

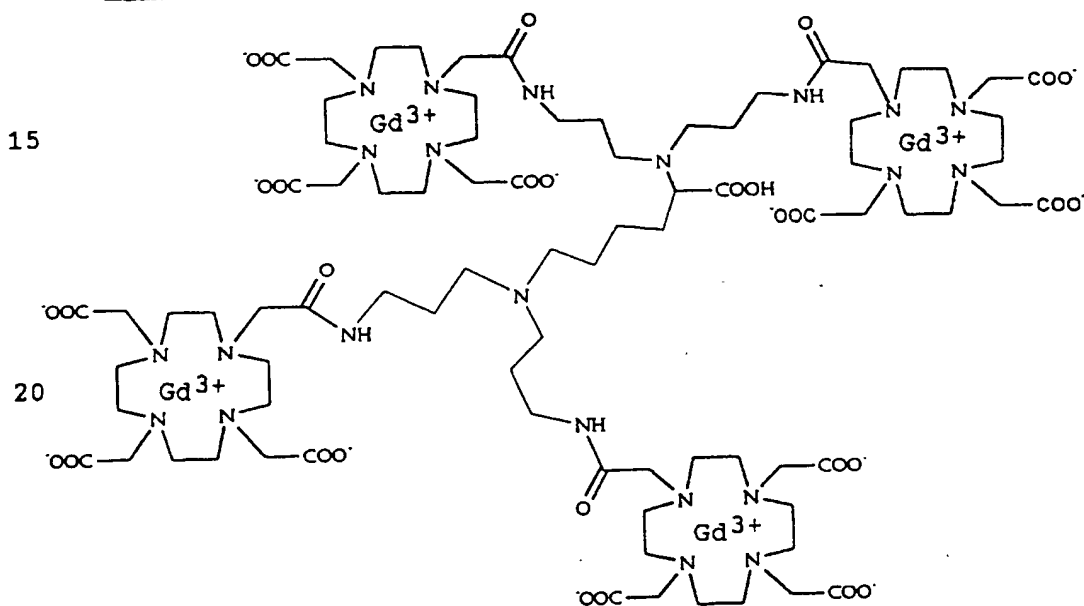
5 MALDI-TOF MS: 1943 (MH^+ calculated 1940).

Relaxivity $r_1 = 5.25 \text{ s}^{-1} \text{ mM}^{-1}$ on a per gadolinium basis.

The ratio for the product described in this example is 1.5.

EXAMPLE 6

10 Gadolinium complex of N^d, N^d, N^E, N^E -tetrakis-[4-aza-5-oxo-6-(1,4,7,10-tetraazacyclododecyl)-4,7,10-triacetate]he-xyl-L-lysine



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2 g of compound (IVa) obtained as described in Example 2B (3.25 mmol) are dissolved in 10 mL methanol and cooled to -5°C in a flask equipped with a reflux condenser. A solution of 75 mg lysine.HCl (0.41 mmol) and 20 mg LiOH (0.82 mmol) in 3 mL methanol and then 310

30 mg of NaCNBH_3 (4.93 mmol) are added. After stirring at

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-5°C for 4 h, the mixture is left under constant stirring at ambient temperature (21°C) for a further 40 h. A further 0.5 g of compound (IVa) (0.82 mmol) dissolved in 2 mL methanol and 77 mg of NaCNBH₃ (1.23 mmol) are then added. This is then heated for 2 h at 50°C and then left at ambient temperature for 17 h. Three mL of H₂O are added to dissolve the residue formed and this is again heated to 50°C for 3 h, before being left for 18 h at ambient temperature. The solvent is evaporated and the residue taken up in 5 mL H₂O, filtered and purified on an ion exchange resin (AG-50W-X4, Bio-Rad) equilibrated in 0.1 M NH₄HCO₃. After removing excess product (IVa) and by-products of the reaction from the column, the product of interest is eluted with a 0.1-2 M gradient of NH₄HCO₃.

The fractions containing the tri- and tetra-alkylation products (TLC: Silica 60-F254, Eluent: ethanol/25% ammonia 1:1; R_f=0.36) are combined, dried and taken up in 3 mL water. This is then purified by preparative HPLC on a LiChrosorb RP-18 (250 x 25 mm) column, eluting the product with a 5 to 20% gradient of acetonitrile containing 0.1% TFA. After a few fractions containing the trisubstituted product, the fractions containing the desired product are collected. These are dried, taken up in water and lyophilised extensively. Yield: 90 mg. MALDI-TOF MS: 2543 (MH⁺ calculated 2538). Relaxivity $r_1 = 8.35 \text{ s}^{-1} \text{ mM}^{-1}$ on a per gadolinium basis.

The ρ ratio for the product described in this example is 2.

30

EXAMPLE 7

N^α-carbobenzyloxy-N^ε,N^ε-bis-[4-aza-5-oxo-6-(1,4,7,10-tetraazacyclododecyl-4,7,10-triacetate)hexyl]-L-lysine

Three g of compound (IIIa) obtained as described in
5 Example 2A (6.5 mmol) and 2 g triethylamine are
dissolved in 10 mL of methanol and then cooled to -5°C,
in an atmosphere of nitrogen. Separately, a solution of
0.45 g of N^α-carbobenzyloxy-L-lysine (Z-lysine, 1.6
mmol) and 0.1 g KOH are prepared in 4 mL of MeOH. This
10 is then added to the flask. Finally 0.6 g of NaCNBH₃
(9.5 mmol) are added and the mixture maintained under
constant stirring at -5°C for 18 h. The solvent is
evaporated and the residue taken up with 10 mL of a
mixture of CH₃CN/H₂O/trifluoroacetic acid (TFA)
15 10:90:0,1. This is filtered to remove the insoluble
residue and purified on a LiChrosorb RP-18 25x250 mm
column (E. Merck) eluting the product isocratically with
the same CH₃CN/H₂O/TFA mixture. The homogenous fractions
are combined on the basis of an analysis carried out
20 under the same conditions on the analytical column and
then evaporated to dryness. The residue is washed with
ether and dried under vacuum. 1.3 g of the desired
product is obtained containing traces of TFA.

K.F. titre: 4.85% (w/w)

25 HPLC titre: 96% (in area %) (method as in Example 3)

ESI-MS spectrum: 1168 (MH⁺)

The following derivatives have been obtained in an
analogous way:

- N^α,N^ε,N^ε(α)-tris-[4-aza-5-oxo-6-(1,4,7,10-tetra-
30 azacyclododecyl-4,7,10-triacetate)hexyl]-L-lysine
- N^α,N^α,N^ε,N^ε-tetrakis-[4-aza-5-oxo-6-(1,4,7,10-te-

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traazacyclododecyl-4,7,10-triacetate)hexyl]-L-lysine

Subjecting the product described in the present example to hydrogenation gives the following derivative:

5 N^E,N^E-bis-[4-aza-5-oxo-6-(1,4,7,10-tetraazacyclododecyl-4,7,10-triacetate)hexyl]-L-lysine.

EXAMPLE 8

Gadolinium complex of 1,1,4,7,7-pentakis-[4-aza-5-oxo-6-(1,4,7,10-tetraazacyclododecyl-4,7,10-triacetate)hexyl]-
10 1,4,7-triazaheptane

1.5 g of compound (IVa) obtained as described in Example 2B (2.44 mmol) are dissolved in 10 mL of 0.1 M boric acid and then 11 μ L of diethylenetriamine (0.1 mmol) are added; the final pH of this solution is 8.5
15 and does not require further adjustment. Sodium cyanoborohydride (300 mg, 4.8 mmol) is then added and the reaction mixture is stirred under nitrogen for 6 h at room temperature and for 60 h at 37°C. At this point the turbid reaction mixture is transferred into a
20 dialysis membrane (Cellusep H1, nominal cut-off 1000 obtained from Membrane Filtration Products, Inc.) and extensively dialysed against water. Mass spectrometry analysis showed the presence of two main peaks at about 2500 and 3100, corresponding respectively to diethylene-
25 triamine-(compound IV)₄ and diethylenetriamine-(compound IV)₅. The dialysed reaction mixture was lyophilised, dissolved in 1 mL of 50 mM phosphate buffer pH 5.5, 0.1 M Na₂SO₄ and loaded on a 2.2x120 cm column of Sephadex G-25, equilibrated in the same buffer. The first peak
30 was collected, concentrated by lyophilisation, dialysed against water and finally lyophilised, yielding 90 mg of

44

the fully alkylated derivative (MH^+ found and calculated = 3092).

EXAMPLE 9

Gadolinium complex of N,N-bis-[4-aza-5-oxo-6-(1,4,7,10-tetraazacyclododecyl-4,7,10-triacetate)hexyl]-dodecyl-amine

2 g of compound (IVa) obtained as described in Example 2B (3.25 mmol) are dissolved in 10 mL of methanol and cooled to $-5^{\circ}C$, in a nitrogen atmosphere. A solution of 125 μ L dodecylamine (100 mg; 0.54 mmol) in 2 mL methanol and then 310 mg of sodium cyanoborohydride (4.9 mmol) are added and the reaction mixture is stirred under nitrogen for 6 h at room temperature and for 60 h at $37^{\circ}C$. The solvent is evaporated and the residue taken up with 10 mL of a mixture of CH_3CN/H_2O /trifluoroacetic acid (TFA) 20:80:0,1. This is filtered to remove the insoluble residue and purified on a LiChrosorb RP-18 25x250 mm column (E. Merck) eluting the product isocratically with the same CH_3CN/H_2O /TFA mixture. The product elutes in a pure form after approximately four column volumes, while impurities are unretarded. The solvent is evaporated to dryness, the residue is washed with ether and dried under vacuum. 450 mg of the desired product is obtained containing traces of TFA.

K.F. titre: 3.97% (w/w)

HPLC titre: 92% (in area %) (method as in Example 3, gradient 10-50%B)

MALDI-TOF-MS spectrum: 1384 (calculated MH^+ 1382)

EXAMPLE 10

Procedure for the conjugation of compound (IVa) with [Lys³]-bombesin.

30 mg of compound (IVa) obtained as described in
5 Example 2B and 5 mg of NaCNBH₃ are added to a solution
of 5 mg of [Lys³]-bombesin (Sigma n. B1647, M_r 1591.8)
in 5 mL of 0.1 M borate buffer pH 8. After 4-h stirring
at room temperature a further 30 mg of compound (IVa)
and 5 mg of NaCNBH₃ are added. After a further 20-h
10 stirring, the reaction mixture is dialysed against water
in a Cellusep H1 membrane (nominal cut-off 1000). The
resulting solution is concentrated by partial
lyophilisation and analysed by mass spectrometry, which
showed the presence of a strong signal at 2790,
15 corresponding to the double alkylation of the single
peptide amino group by compound (IVa) (calculated
MH⁺=2788).

EXAMPLE 11

Procedure for the conjugation of compound (IVa) with
20 insulin.

95 mg of compound (IVa) obtained as described in
Example 2B and 15 mg of NaCNBH₃ are added to a solution
of 20 mg of porcine insulin, sodium salt (Calbiochem n.
407696, M_r 5778; 10 µmol amino groups) in 13 mL of
25 borate buffer 0.1 M, pH 8. After 4 h of reaction at 20°C
a further 95 mg of compound (IVa) and 15 mg of NaCNBH₃
are added.

The conjugation reaction takes place at a total
molar ratio of 30:1 of compound (IVa) with respect to
30 the amino groups present and at 1.5:1 between the
NaCNBH₃ and compound (IVa). After a further 16 h at

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ambient temperature, the conjugate is separated from the excess of reagent and from the by-products of reaction by size exclusion chromatography on a Sephacryl S-100HR column (Pharmacia). The column (45 x 8.9 cm) is eluted with 0.15 M NH_4HCO_3 at 20 mL/min. The final product is concentrated and desalted by ultrafiltration/diafiltration on an Amicon YM-3 membrane.

The product is homogenous as assessed by size exclusion chromatography ($t_R = 24.99$ min). Treatment with fluorescamine has revealed the presence of only 0.1 mol NH_2 groups/mol protein. The Gd content is 4.6 mol/mol protein. Mass spectrometry has revealed signals corresponding to the mass of insulin plus, respectively, 2, 3, 4 and 5 residues of compound (IVa). Amino acid analysis (see following Table 2) reveals excellent agreement with the composition of non-modified insulin, with the exception of the amino acids lysine, glycine and phenylalanine which are reduced by one unit. Since the latter two amino acids are the amino terminals of the two chains of insulin, this confirms that both the α - and ϵ - amino groups take part in the reaction. The low quantity of cysteine found is due to the typical instability of this amino acid under hydrolytic conditions and has not been considered.

47

Table 2. Analysis of the amino acids of the insulin-compound (IVa) conjugate

| ----- | | | | |
|-------|-------------|-------|--------------------------|-------|
| | Theoretical | Found | D (Found Theoretical) | |
| ----- | | | | |
| 5 | | | | |
| | Asx | 3 | 3.04 | 0.04 |
| | Thr | 2 | 1.94 | -0.06 |
| | Ser | 3 | 2.70 | -0.30 |
| 10 | Pro | 1 | 1.27 | 0.27 |
| | Glx | 7 | 7.29 | 0.29 |
| | Gly | 4 | 3.09 | -0.91 |
| | Ala | 2 | 1.99 | -0.01 |
| | Cys | 6 | 4.53 | -1.47 |
| 15 | Val | 4 | 4.30 | 0.30 |
| | Met | - | - | |
| | Ile | 2 | 1.87 | -0.13 |
| | Leu | 6 | 5.97 | -0.03 |
| | Tyr | 4 | 3.92 | -0.08 |
| 20 | Phe | 3 | 2.09 | -0.91 |
| | His | 2 | 1.99 | -0.01 |
| | Lys | 1 | 0.17 | -0.83 |
| | Arg | 1 | 0.99 | -0.01 |

25 The ρ ratio for the product described in this example is 1.59.

EXAMPLE 12

Procedure for the conjugation of compound (IVa) with myoglobin.

30 The procedure is as for Example 11, using horse heart myoglobin (commercially available from Sigma, n. M-1882, M_r 17567) and borate buffer pH 8.5. The

quantities used are:

0.5 g myoglobin (0.56 mmol NH_2) in 100 mL buffer
10.37 g compound (IVa) obtained as described in Example
2B added in two lots (16.9 mmol)
5 1.6 g NaCNBH_3 added in two lots (25.4 mmol).

The product has been shown to be homogenous upon
analysis by size exclusion chromatography ($t_R = 19.58$
min). Treatment with fluorescamine has revealed the
presence of only 0.5 mol NH_2 groups/mol protein. The Gd
10 content is 27.9 mol/mol protein. Mass spectrometry has
revealed signals corresponding to the mass of myoglobin
plus, respectively, from 29 to 33 residues of compound
(IVa).

Amino acid analysis, used to determine the protein
15 content of the prepared compound, has shown that the
composition of the treated myoglobin is in excellent
agreement with the composition of non-modified
myoglobin, with the exception of lysine, which, instead
of there being 19 residues, was shown to be next to
20 zero.

Relaxivity $r_1 = 19 \text{ s}^{-1} \text{ mM}^{-1}$ on a per gadolinium
basis.

The ρ ratio for the product described in this
example is 1.43.

25 EXAMPLE 13

Procedure for the conjugation of compound (IVa) with
chymotrypsinogen A.

The procedure is as described for Example 11, using
bovine pancreas chymotrypsinogen A (commercially
30 available from E. Merck, n. 2306, M_r 25656) and borate
buffer pH 9. The quantities used are:

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0.5 g chymotrypsinogen A (0.29 mmol NH_2) in 100 mL buffer

5.38 g compound (IVa) obtained as described in Example 2B added in two lots (8.77 mmol)

5 0.82 g NaCNBH_3 added in two lots (13.2 mmol).

The product has been shown to be homogenous upon analysis by size exclusion chromatography ($t_R = 19.55$ min). Treatment with fluorescamine has revealed the presence of only 0.2 mol NH_2 groups/mol protein. The Gd content is 28.2 mol/mol protein. Mass spectrometry has revealed a widened signal whose centre corresponds to the mass of chymotrypsinogen A plus 26.6 residues of compound (IVa).

Amino acid analysis, used to determine the protein content of the prepared compound, has shown that the composition of the treated chymotrypsinogen A is in excellent agreement with the composition of non-modified chymotrypsinogen A, with the exception of lysine, which, instead of there being 14 residues, was shown to be next to zero.

The ρ ratio for the product described in this example is 1.91.

EXAMPLE 14

Procedure for the conjugation of compound (IVa) with cytochrome c.

The procedure is as for Example 11, using horse heart cytochrome c (commercially available from Sigma, n. C-7752, M_r 12360). The quantities used are:

20 mg cytochrome c (32 μmol NH_2) in 10 mL buffer

30 0.55 g compound (IVa) obtained as described in Example 2B added in two lots (0.9 mmol)

50

100 mg NaCNBH_3 added in two lots (1.6 mmol).

Prior to purification by column, the reaction mixture is first dialysed against borate buffer to remove unreacted NaCNBH_3 and then treated with potassium ferricyanide (final concentration 5 mM) to reoxidise the ferroion of the heme group.

The product has been shown to be homogenous upon analysis by steric exclusion chromatography. As it was not possible to treat the product with fluorescamine because of interference on the part of the heme group, the free amino groups were determined with TNBS (Habeeb A.F.S.A. Anal. Biochem., 1966, 14, 328-336) which gave a value of 9 mol NH_2 groups/mol protein. The Gd content was 14.6 mol/mol protein. Mass spectrometry revealed a widened signal in which 3 peaks were visible corresponding to the mass of cytochrome c plus 15, 16 and 17 residues of compound (IVa).

The P ratio for the product described in this example is 1.46.

20 EXAMPLE 15

Procedure for the conjugation of compound 2A) with insulin.

Compound 2A), 70 mg, (0.155 mmol) is added to a solution of 10 mg of porcine insulin, sodium salt (Calbiochem n. 407696, M_r 5778; 5.2 μmol amino groups) in 7 mL of borate buffer 0.1 M pH 8, with the pH maintained at 8 with 1 N NaOH. 15 mg of NaCNBH_3 are then added and the mixture left under constant stirring at ambient temperature for 20 h.

30 The reaction mixture is concentrated by ultrafiltration (Amicon YM-3 membrane) and then loaded

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on a Sephacryl S-100HR column (Pharmacia). The column (2.2 x 100 cm) is eluted with 0.15 M NH_4HCO_3 at 1 mL/min. The final product is concentrated and desalted by ultrafiltration /diafiltration on an Amicon YM-3 membrane.

The product has been shown to be homogenous upon analysis by steric exclusion chromatography.

Treatment with fluorescamine has revealed the presence of only 0.3 mol NH_2 groups/mol protein.

Mass spectrometry has revealed signals corresponding to the mass of insulin plus, respectively, 2, 3, and 4 residues of compound 2A).

By integration of the signals the mean number of substituents is calculated to be 3.4. The ρ ratio for the product described in this example is 1.26.

The following derivatives were obtained using the same procedure:

- [Formula III]myoglobin
- [Formula III]cytochrome c
- [Formula III]chymotrypsinogen A

EXAMPLE 16

Procedure for the conjugation of compound 2A) with polylysine.

1 g of compound 2A) (1.6 mmol) is added to a solution of 10 mg of polylysine bromohydrate (Sigma n. P0879, M_r 1000-4000; 52 μmol amino groups, assuming a mean of 10 Lys residues and M_r 2108, based on MALDI-TOF MS analysis) in 7 mL of borate buffer, 0.1 M pH 8, with the pH maintained at 8 with 1 N NaOH. 150 mg of NaCNBH_3 are then added and the mixture left under constant stirring for 72 h at ambient temperature.

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The reaction mixture is then loaded on a Sephacryl S-100HR column (Pharmacia). The column (8.9 x 45 cm) is then eluted with 0.15 M NH_4HCO_3 at 20 mL/min. The first peak is concentrated by evaporation and then lyophilised (52 mg).

Mass spectrometry (MALDI-TOF) has revealed a wide distribution of signals in the region between 7000 and 13000, centred around 9600 which corresponds to the mean mass of polylysine plus 14 units of compound 2A).

Treatment with fluorescamine has revealed the presence of only 1.2 mol NH_2 groups/mol protein, assuming $M_r = 9600$.

CLAIMS

1. Polychelant/polychelate compounds and their physiologically compatible salt comprising an organic backbone carrying "m" primary amino groups, where m is a number from 1 to 1000, in which said amino groups are alkylated with "n" chelant/chelate residues, where n is a number from 2 to 2m, said chelant/chelate residues being covalently linked to said amino groups by means of an aliphatic chain, with this chain interrupted or not by heteroatoms selected from O, N, S and/or by groups selected from carbonyl, thiocarbonyl, amide, ester, thiourea and thioamide groups or aromatic groups, and in which a number "p" of said amino groups, where p is a number from 0 to m-1, is non-alkylated, said compounds being characterized by the fact that at least one of said primary amino groups is di-alkylated with two of said chelant/chelate residues and that the parameter ρ , which represents the numerical ratio between the number n of said linked chelant/chelate residues and the number m-p of the alkylated amino group, is greater than 1 and not greater than 2, i.e.

$$1 < \rho \leq 2$$

2. Compounds of general formula:



in which

L is an organic backbone

F is a $-(CH_2)_q-T-K$ residue

where

T is a simple bond or an aliphatic chain, interrupted or not by one or more heteroatoms selected from

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O, N, S or by functional groups selected from carbonyl, thiocarbonyl, amide, ester, thiourea, thioamide groups or aromatic residues, said chain being linked covalently to an atom of C, O, N, or P of a residue K,

K is the residue of a linear or cyclic polyaminopolycarboxylic or polyaminopolyphosphonic or polyaminopolyphosphoric, or polyamino polyphosphinic chelant, or one of their metal chelates, or one of their salts,

q is an integer from 1 to 10

p is a number from 0 to m-1

z is a number from 0 to m-1

x is a number from 1 to m

where m is a number from 1 to 1000, being m the total number of the primary amino groups originally present on L,

with the condition that $p + x + z = m$, and in which the chelated metal ions are bi- or trivalent paramagnetic ions or radioisotopes.

3. Compounds according to claim 2, in which:

- L is selected from a group constituted by: spermidine, norspermidine, spermine, 4,9-dioxadodecandiamine, 3,6-dioxaoctandiamine, ethanolamine and homologues, phosphatidylethanolamine and its derivatives, sphingosine, alkylamines, alkylenediamines, diethylenetriamine, triethylenetetramine, tris-(2-aminoethyl)amine, jeffamine, N-glucosamine, lysine and derivatives, ornithine, glycine, aminobutyric acid, aminocaproic acid, taurine and its derivatives, [Lys³]-bombesin,

55

sinsulin, chymotrypsinogen A, myoglobin, albumin, cytochrome c, branched and linear polylysine, branched and linear polyornithine, amino sugars, polypeptides, hormones, growth factors, antibodies;

5 T is a simple bond or an aliphatic chain, containing an ester, amide or carbonylamino group, said chain being linked covalently to a nitrogen or carbon atom of a K residue,

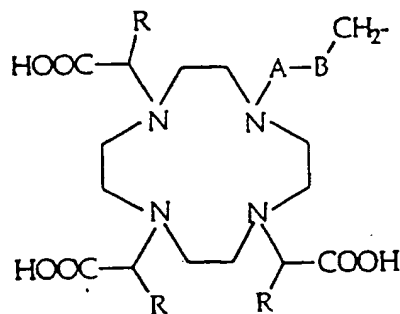
K is the residue of a polyaminopolycarboxylic acid selected from the group comprising: EDTA, DTPA, BOPTA, EOB-DTPA, DOTA, their derivatives, metal chelates or salts,

- the metal ion chelates are selected from the bi- or trivalent ions of elements having atomic numbers between 20 and 31, or 39, 42, 43, 44, 49, or between 57 and 83 or the ions of the following radioisotopes ^{51}Cr , ^{67}Ga , ^{68}Ga , ^{111}In , $^{99\text{m}}\text{Tc}$, ^{140}La , ^{175}Yb , ^{153}Sm , ^{166}Ho , ^{90}Y , ^{149}Pm , ^{177}Lu , ^{47}Sc , ^{142}Pr , ^{159}Gd , ^{212}Bi .

20 4. Compounds according to claim 3, in which the said metal ions are selected from $\text{Mn}(2+)$, $\text{Fe}(3+)$, $\text{Gd}(3+)$, $\text{Eu}(3+)$, $\text{Dy}(3+)$, $^{111}\text{In}(3+)$, $^{99\text{m}}\text{Tc}(3+)$.

5. Compounds according to claims 2-4, in which F is a chelant residue of formula (I)

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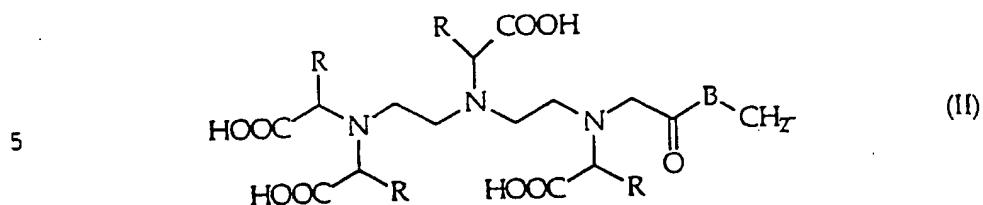


(I)

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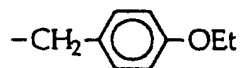
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or formula (II)



in which

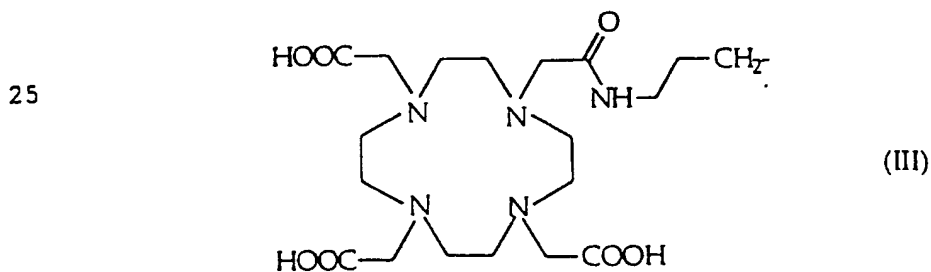
- 10 A corresponds to a simple bond or to a $-(CH_2)_sR_1-$ group where s is an integer from 1 to 5 and R_1 is a simple bond or is equal to CONH, NHCO, NHCSNH, $C_6H_4NHCSNH$, COO, OCO, O, S,
- B corresponds to $-D-(CH_2)_t-$, in which t is an integer from 1 to 9 and
- 15 D is a simple bond, or is equal to $-O-$, $-NH-$,
- R in each compound can be H or CH_3 or CH_2-O-Bz or



or any combination of them,

20 as well as their metal chelates, or their salts.

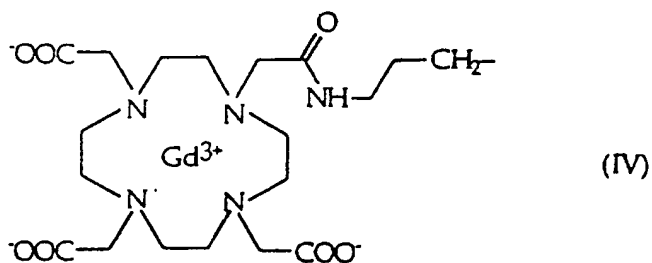
6. Compounds according to claim 5, in which F is a residue of formula (III) or (IV)



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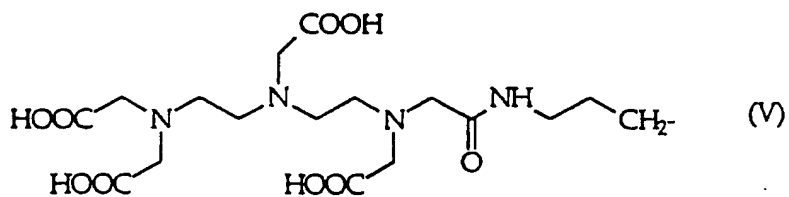
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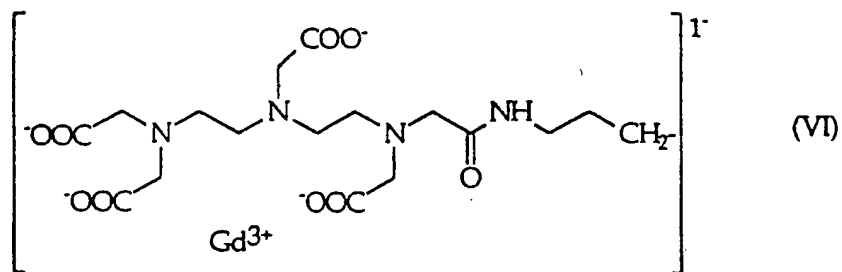


or of formula (V) or (VI)

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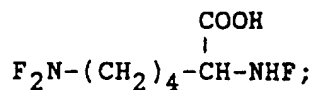
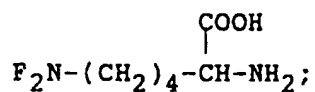
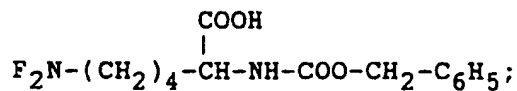


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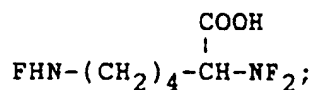
or one of their salts.

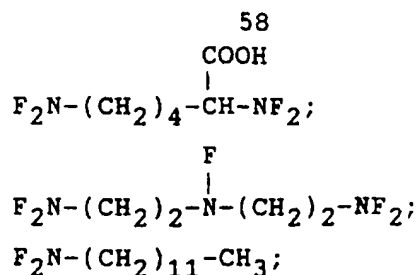
7. Compounds according to claim 6, selected from the group comprising:

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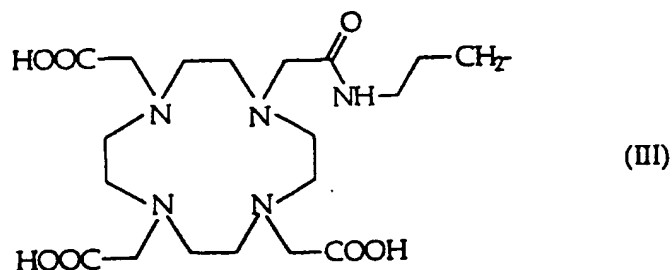


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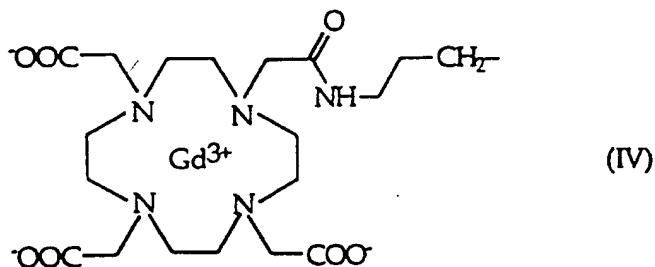




where F is a chelant residue of formula (III)



or a chelate of formula (IV)



or a salt thereof.

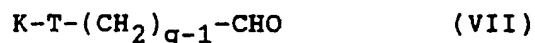
8. Compounds according to claims 3 and 6, in which F is a residue of formula (III) or formula (IV) and L is selected from the group comprising [Lys³]-bombesin, insulin, myoglobin, albumin, cytochrome c, chymotrypsinogen A, polylysine.
9. A process for the preparation of compounds of claim 1 which includes functionalisation by means of reductive

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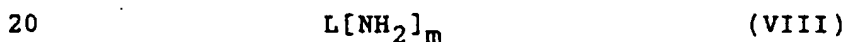
alkylation of one or more primary amino groups of the organic backbone with an aldehyde linked to a chelant residue, or to its chelate, or salt, by means of an aliphatic chain as defined in claim 1, characterised by the fact that the aldehyde is in a 3 to 40 fold molar excess over the total number of primary amino groups.

10. A process for the preparation of compounds of claim 1 which includes the alkylation of one or more primary amino groups of the organic backbone with an alkylene halide linked to a chelant residue, or to its chelate, or salt, by means of an aliphatic chain as defined in claim 1.

11. A process for the preparation of compounds of claims 2-6 which includes the reaction of a chelant compound of formula (VII)



where K, T, q are as described above, or a chelate, or a salt thereof, with a polyamino compound of formula (VIII)



where L and m are as described above, in a reaction medium, under conditions of reductive alkylation, characterised by the fact that the compound of formula (VII) is in a 3 to 40 fold molar excess over the number m of primary amino groups, and occurs in the presence of a reducing agent specific for the imine bond, but not for the aldehyde, said reducing agent being in a 3-60 fold excess with respect to the total number of primary amino groups.

12. A process according to claim 11, in which said reaction medium is selected from aqueous buffer, at

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pH from 5 to 10, low molecular weight alcohols, - an aprotic dipolar solvent, or even a mixture of the same.

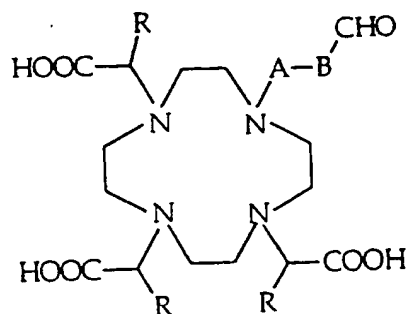
13. A process according to claim 11 or 12, in which the reaction temperature is between -5 and 60°C for a length of time of between 2 and 170 h.

14. A process according to claims 11-13, in which:

- a chelate of formula (VII), or one of its salts, is employed in a molar excess of about 10-35 fold with respect to the total number of amino groups;
- the reaction medium is an aqueous buffer at a pH of 7-9, or methanol, or a mixture of the two;
- the reducing agent is sodium cyanoborohydride;
- the temperature is variable between 15-30°C;
- the reaction time is between 10-72 h.

15. A process according to claims 11-13, in which the reductive alkylation reaction occurs between a chelant of formula (VII) and a polyamino residue $L(NH_2)_m$, followed by the subsequent formation of the relative metal complex and/or one of its salts.

16. A process for the preparation of intermediates of formula (Ia)



(Ia)

and of the metal chelates and salt thereof,
in which

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A corresponds to a simple bond or to a $-(CH_2)_sR_1-$ group where s is an integer from 1 to 5 and

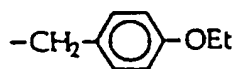
R_1 is a simple bond or is equal to CONH, NHCO, NHCSNH, $C_6H_4NHCSNH$, COO, OCO, O, S,

5 B corresponds to $-D-(CH_2)_t-$, in which

t is an integer from 1 to 9 and

D is a simple bond, or is equal to $-O-$, $-NH-$,

R in each compound can be H or CH_3 or CH_2-O-Bz or



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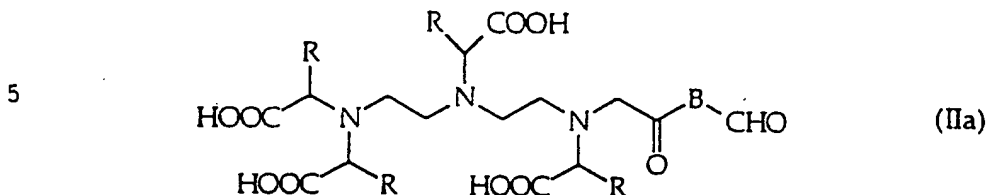
or any combination of them

and which includes the following steps:

- preparation of the protected aldehyde X-A-B-Y in which A and B are as defined above, X is a leaving group selected from the halogens, OTs, OMs, OTf, and Y is the aldehyde protected with a protective group labile in an acid environment, such as a derivative of 1,3-dioxolane and 1,3-dioxane;
- 15 - reaction between TAZA and the X group of said protected aldehyde to give the corresponding 1:1 condensation product;
- condensation with α -R-bromoacetic acid, in which R is defined as above, and deprotection of the aldehyde function;
- 20 - formation of the metal complex and/or its salt by means of reaction of the chelant of formula (Ia) obtained in the previous step, with a metal in the form of a salt or oxide and in the presence or absence of the quantity of base or acid necessary to obtain a neutral salt.
- 25
- 30

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17. A process for the preparation of intermediates of formula (IIa)



and of the metal chelates and salt thereof,
in which:

B corresponds to $-D-(CH_2)_t-$, in which
t is an integer from 1 to 9 and
D is a simple bond, or is equal to $-O-$, $-NH-$,
R in each compound can be H or CH_3 or CH_2-O-Bz or



or any combination of them,

and which includes the following steps:

- preparation of the protected aldehyde X-B-Y where X represents a leaving group, Y is the protected aldehyde and B is as defined above;
- transformation of X into the amine to give the protected aldehyde H_2N-B-Y ;
- condensation between the dianhydride of the R derivative of the diethylenetriaminepentaacetic acid with the protected aldehyde prepared previously;
- deblocking of the protected aldehyde;
- formation of the complex.

18. Pharmaceutical and/or diagnostic compositions including as active ingredient at least one of the

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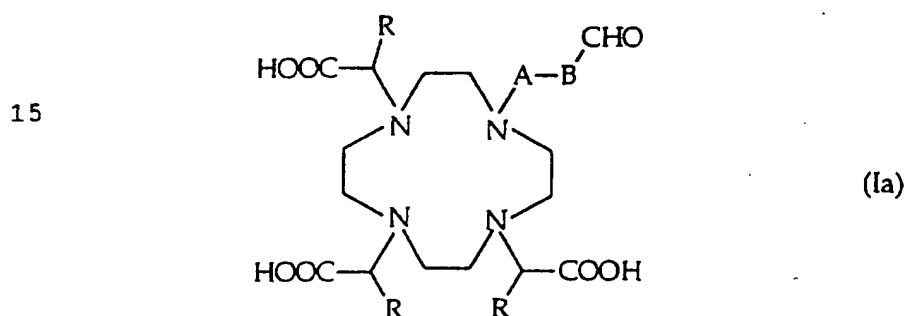
compounds of claims 1-8, or one of their physiologically compatible salts.

19. The use of the compounds of claims 1-8, in the form of a physiologically compatible chelate or salt, for the preparation of pharmaceutical compositions for use in radiotherapy.

20. The use of the compounds of claims 1-8, in the form of a physiologically compatible chelate or salt, for the preparation of contrast media for NMR imaging.

21. The use of the compounds of claims 1-8 for the preparation of contrast media for scintigraphy.

22. Compounds of formula (Ia):



20 in which

A corresponds to a simple bond or to a $-(CH_2)_sR_1-$ group where s is an integer from 1 to 5 and

R_1 is a simple bond or is equal to CONH, NHCO, NHCSNH, $C_6H_4NHCSNH$, COO, OCO, O, S,

25 B corresponds to $-D-(CH_2)_t-$, in which t is an integer from 1 to 9 and

D is a simple bond, or is equal to $-O-$, $-NH-$,

R in each compound can be H or CH_3 or CH_2-O-Bz or

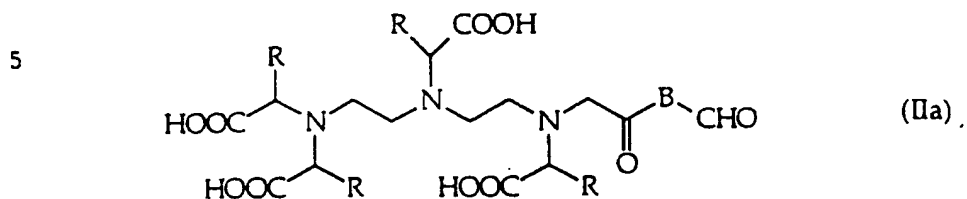


or any combination of them,

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for use as intermediates for the preparation of compounds of claims 5-8.

23. Compounds of formula (IIa)



in which:

- 10 B corresponds to $-D-(CH_2)_t-$, in which
 t is an integer from 1 to 9 and
 D is a simple bond, or is equal to $-O-$, $-NH-$,
 R in each compound can be H or CH_3 or CH_2-O-Bz or



or any combination of them,
 for use as intermediates for the preparation of
 compounds of claims 5-8.

INTERNATIONAL SEARCH REPORT

Int. Appl. No.
PCT/EP 97/01048

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|---|--|---|
| A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C07D257/02 C07C229/16 A61K49/00 | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C07D C07C A61K | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | FR 2 596 992 A (GUERBET SA.) 16 October 1987 see claims --- | 1-23 |
| A | EP 0 661 279 A (GUERBET SA.) 5 July 1995 see claims --- | 1-23 |
| A | WO 95 27705 A (BRACCO INTERNATIONAL B.V.) 19 October 1995 see claims ----- | 1-23 |
| <div style="display: flex; justify-content: space-between;"> <input type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div> | | |
| <div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> | | |
| Date of the actual completion of the international search <div style="text-align: center;">16 May 1997</div> | | Date of mailing of the international search report <div style="text-align: center;">27.05.97</div> |
| Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016 | | Authorized officer <div style="text-align: center;">Chouly, J</div> |

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